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# CRYOPRESERVATION OF INTACT HUMAN ARTICULAR CARTILAGE

by

**NADR MOHAMED JOMHA** ©

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The undersigned certify that they have read, and recommend the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **CRYOPRESERVATION OF INTACT HUMAN ARTICULAR CARTILAGE** submitted by **Nadr Mohamed Jomha** in partial fulfillment of the requirements for the degree of **Master of Science** in **Experimental Surgery.**

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# ABSTRACT

Although still evolving, cryopreservation and transplantation applications in Orthopedic surgery involve cryopreserving intact human articular cartilage (AC) in the form of osteochondral grafts for treatment of bone tumors, joint trauma, and arthritis. Basic research has utilized individual cell lines and animal models but little work has been done with intact cartilage from humans. Initial research has developed potential techniques but ultimately studies must be performed on human models. Four studies were performed using intact human AC to examine basic concepts as applied to humans. These studies attempted to validate techniques on animal models and improve on these techniques. Further work defined causes of chondrocyte damage and documented chondrocyte morphology while undergoing these techniques. Cellular evaluation included vital dye staining and scanning electron microscopy. The results of these studies enhanced the understanding of events associated with cryopreservation of human AC and provided a scientific basis for future study in this area.

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# TABLE OF CONTENTS

CHAPTER PAGE

1. CRYOPRESERVATION INTRODUCTION

1.1 General Introduction 2
1.2 Indications for Articular Cartilage Transplantation 3
1.3 Mechanism of Cryopreservation 11
1.4 Cryopreservation of Articular Cartilage 19
1.5 Cryoprotectants 23
1.6 Cell Recovery 27
1.7 Research Introduction 32
1.8 References 34

2. CRYOPROTECTION OF INTACT HUMAN ARTICULAR CARTILAGE

2.1 Abstract 44
2.2 Introduction 45
2.3 Methods and Materials 48
2.4 Results 50
2.5 Discussion 52
2.6 Acknowledgments 57
2.7 References 58
2.8 Tables and Figures 64

3. CRYOPROTECTION SOLUTIONS FOR INTACT HUMAN ARTICULAR CARTILAGE

3.1 Summary 69
3.2 Introduction 71
3.3 Methods and Materials 74
3.4 Results 76
3.5 Discussion 77
3.6 Acknowledgments 82
3.7 References 83
3.8 Tables and Figures 88

4. LOCALIZATION OF CHONDROCYTE INJURY DURING CRYOPRESERVATION OF INTACT HUMAN ARTICULAR CARTILAGE

4.1 Abstract 94
4.2 Introduction 96
4.3 Methods and Materials 97
4.4 Results 99
4.5 Discussion 100
4.6 Acknowledgments 108
4.7 References 109
4.8 Table 116

5. MORPHOLOGY OF HUMAN ARTICULAR CARTILAGE WITH CRYOPRESERVATION AND FREEZING

- 5.1 Abstract 119
- 5.2 Introduction 121
- 5.3 Methods and Materials 123
- 5.4 Results and Discussion 125
- 5.5 Acknowledgments 134
- 5.6 References 135
- 5.7 Figures 141

6. CONCLUSION

- 6.1 Conclusion 152

TABLES PAGE

Table 2.1 Chondrocyte recovery 64

Table 3.1 Tukey's test for chondrocyte recovery from -196°C 88

Table 3.2 Tukey's test for chondrocyte recovery from -30°C 89

Table 4.1 Chondrocyte recovery from various conditions 116

FIGURES PAGE

Figure 2.1 Chondrocyte recovery from PBS directly warmed 65

Figure 2.2 Chondrocyte recovery from PBS in LN 65

Figure 2.3 Chondrocyte recovery from $Me_2SO$ directly warmed 66

Figure 2.4 Chondrocyte recovery from $Me_2SO$ in LN 66

Figure 3.1 Chondrocyte recovery from -30ºC 90

Figure 3.2 Chondrocyte recovery from -196ºC 91

Figure 5.1 SEM - fresh control in PBS 141

Figure 5.2 SEM - fresh control in 1,2-propanediol 142

Figure 5.3 SEM - negative control 143

Figure 5.4 SEM - PBS at -5ºC 144

Figure 5.5 SEM - PBS at -5ºC 145

Figure 5.6 SEM - 1,2-propanediol at -5ºC 146

Figure 5.7 SEM - 1,2-propanediol at -5ºC 147

Figure 5.8 SEM - PBS at -30ºC 148

Figure 5.9 SEM - $Me_2SO$ at -30ºC 149

Figure 5.10 SEM - 1,2-propanediol at -30ºC 150

ABBREVIATIONS

| | |
|---|---|
| AC | articular cartilage |
| ANOVA | analysis of variance |
| BME | basal medium eagle |
| DNA | deoxyribonucleic acid |
| EB | ethidium bromide |
| FDA | fluorescein diacetate |
| GLUC | glucose |
| H and E | hematoxylin and eosin |
| HES | hydroxyethyl starch |
| LN | liquid nitrogen |
| $Me_2SO$ | dimethyl sulfoxide |
| METH | methanol |
| min | minute |
| OCD | osteochondral dowel |
| PBS | phosphate buffered saline solution |
| PG | 1,2-propanediol (propylene glycol) |
| PVP | polyvinylpyrrolidone |
| SEM | scanning electron microscopy |
| TEM | transmission electron microscopy |
| TKA | total knee arthroplasty |
| UV | ultraviolet |

# CHAPTER 1

## 1.1 INTRODUCTION

Medical advances in recent years have produced many innovative techniques to treat different diseases. Transplantation of tissues and organs is becoming more popular. Technical advances have provided the opportunity while increased frequency has allowed development of the skill necessary to perform these procedures. Even though transplantation is commonly successful with tissues such as corneas, transplantation of more complex organs such as hearts, kidneys, and articular cartilage have proven more difficult. Problems such as size matching, infectious disease transmission, and rejection are difficult to overcome without significant compromises in quality of life.

Cryopreservation of tissues has been around from the beginning of time (although not necessarily always intentional). Only recently has man attempted to harness the useful effects of excessive cold for medical treatment. In the last two hundred years attempts have been made with only relatively recent success. In the last 50 years cryoprotectants have been added to the equation with resultant increased success at cryopreserving many tissues, initially with sperm, then progressing to other tissues like erythrocytes and embryos. More recently there has been increased interest in cryopreserving more complex tissues such as hearts, kidneys, and articular cartilage.

Transplantation of more complex tissues would be more successful and safer following cryopreservation. Cryopreservation would allow storage of these tissues for long periods of time allowing for better size matches between organ and recipient, more effective infectious disease testing and increased compatibility between donor and recipient. Work in this area has increased due to increased pressure from the medical community to come up with safer, more available options than using fresh donor material presently in use for many organ transplantations.

The related practices of transplantation and cryopreservation have evolved across many medical disciplines involving different services. Orthopedic surgery is one of those services involved. Recently there has been increased interest in transplanting bone as bone graft but also in transplanting articular cartilage. These tissues encounter many of the problems experienced by other complex tissues. The ability to overcome these barriers would enable Orthopedic surgeons to expand current practice techniques for many diseases including bone tumors, joint trauma, and arthritis. Advances in Orthopedic surgery may also be transferable across disciplines and help push back the frontiers of medicine providing valuable options and resources previously unavailable to patients.

## LITERATURE REVIEW

### 1.2 INDICATIONS FOR ARTICULAR CARTILAGE TRANSPLANTATION

There is increased demand for cryopreservation of human articular cartilage (AC) for transplantation for treatment of diseases including osteoarthritis, joint trauma, bone tumors, and osteochondritis dissecans. The ability to cryopreserve AC from human cadaveric donors will allow matching for size and joint contour congruency and screening for transmissible infectious diseases to enhance clinical outcomes of transplantation. The driving force to develop this technology has been an attempt to find an alternative to total joint replacement and provide better function for younger patients with severe joint disorders.

Fresh osteochondral allografts have been used successfully in the past. Czitrom et. al. (9) in 1985 reviewed over 14 years experience in Toronto of transplanting fresh osteochondral fragments into small osteochondral defects in over 100 patients. In the same review were 55 patients with reconstruction of large osteochondral defects due to bone tumors. The best results were in traumatic injuries with poorer results in primary osteoarthritis, osteonecrosis, osteochondritis dissecans, and patients on steroids; single pole grafts did better than bipolar grafts. The authors determined that chondrocytes and hyaline cartilage were maintained up to 8 years in some grafts.

In 1986 Garrett (16) reviewed 24 fresh osteochondral allografts in the treatment of osteochondritis dissecans resistant to conservative treatment in an attempt to prevent severe degenerative changes. The patients were arthroscopically assessed to determine the size of the defect. Skeletal size was measured by standard radiographs of the distal femur as it was felt very important that matching of the defect in all dimensions, including thickness, was essential. Transplantation was done within 12 hours of the harvest of the graft. The host bed was fashioned and an identical osteochondral plug was inserted and pressfit or fixed with K-wires. The size of defects ranged from 1.2cm - 2.75cm in diameter. The results indicated that all 24 patients had improved function, decreased pain and swelling, and no buckling of the knee. In 23/24 patients the graft was healed within 6 weeks. Arthroscopic investigation of 11 knees was performed and only limited fraying and delamination was noted at the periphery of the graft and in the area of articular cartilage immediately adjacent in nine of the 11 patients. No displacement or collapse was noted. All patients improved symptomatically in this series and the author concluded that this procedure may minimize osteoarthritic changes. Graft rejection was not a problem as noted by the excellent healing of the graft to the host.

Convery et. al. (8) also reviewed results, retrospectively, of 37 fresh osteochondral allografts of the femoral condyle and took special note of ones greater than 5 years old. The sizes once again were 1cm x 1cm - 2cm x 6cm and a 5mm thickness of subchondral bone was left attached and cleansed by high pressure lavage. There was no tissue typing, blood group matching or immunosuppression performed. The results of the >5 years old group (total of 12) revealed a dropout of three (two due to unrelated death and one failure due to very poor size matching because of poor graft selection). Eight of the remaining nine knees had excellent to good results. Of the group totaling 37 there were three more failures and all three of these had an anterior cruciate ligament deficient knee. Twenty five of the 37 knees were rated good to excellent in the paper. All failures (four) were mechanical in origin therefore mechanical factors and risk of immune response were felt to be most important for clinical success.

These studies and others (15,58) looked at the possibility of transplanting articular cartilage for joint defects and consistently found allograft matching for size and joint contour of the transplanted cartilage with the defect was extremely important to optimize the results of transplantation and that an inappropriate match would lead to early breakdown of the transplanted cartilage and subsequent failure. Other important factors included joint alignment and stability, defect site, bone resorption and resultant cartilage collapse due to immune response and graft rejection, coexisting joint degeneration, survival of chondrocytes, and age of the donor. The timing of the transplantation was another concern. There was uncertainty with respect to the length of time that fresh AC could endure prior to transplantation before chondrocyte survival began to diminish. Earlier transplantation likely leaves healthier chondrocytes suggesting that transplantation should be done on an emergent basis which is not optimal due to the staffing requirements for these specialized procedures. One more consideration was the possibility of transmission of infectious diseases which can occur (6,63) and an increased risk would be present with transplantation of fresh AC.

These problems led to renewed interest in cryopreservation of AC. The ability to cryopreserve human AC would provide many options to Orthopedic surgeons treating patients with joint diseases. The ability to bank human AC would allow matching for size and joint contour congruency by incorporating standardized radiographs of the donor and recipient into the process thereby allowing for more consistent results. Successful cryopreservation would also allow AC storage and surgery could then be performed on an elective basis with appropriate staff available. Finally, the ability to store the cartilage would allow further testing of donor cartilage for any infectious diseases prior to the transplantation. These procedures would enhance the success rate of the transplantation as well as increase the safety margin of the procedure. With these objectives, investigators have attempted to cryopreserve AC with various protocols. To date there has not been one consistently accepted method and clinical studies continue to examine the results of transplanted cryopreserved human AC.

Early work was done by Schachar et. al. (54) on rabbit AC. Based on previous work showing that individual chondrocytes could maintain viability and ability to grow in culture following cryopreservation, survival and function of transplanted allograft cartilage was evaluated by quantitative assessments of metabolism and biochemical parameters. New Zealand white rabbits were used to support AC autografts and allografts. The allografts were immersed with a cryoprotectant of 7.5% $Me_2SO$, slowly frozen to -70°C and stored for a minimum of one week. The AC was transplanted and the animals were sacrificed at three, six, nine, and 12 months. There were 45 grafts in all (29 allografts and 16 autografts). Joint space congruency and thickness were preserved in all but two joints (one autograft and one allograft). All the grafts healed although the autografts healed more quickly. The gross appearance of the cartilage was normal. Metabolic analysis was performed by observing the $^3H$ uptake indicative of collagen turnover and $^{35}S$ uptake indicative of proteoglycan turnover. Metabolism was present in all grafts up to 12 months but was consistently lower in allografts with a significant difference between allografts and autografts at 12 months. The authors concluded that cryopreserved AC retained its metabolic and biochemical activity and therefore was biologically viable and functional at one year after transplantation. The autografts showed better maintenance and improvement of metabolic and biochemical activity than the allografts after longer time intervals but the allografts did maintain some function up to one year. One problem with radioactive incorporation of metabolites is that chondrocytes from osteoarthritic joints are considerably more metabolically active than normal chondrocytes (48), therefore increased incorporation of radioactive metabolites is unreliable in assessing degree of cell survival.

Malinin (25) investigated the long term results of transplanted cryopreserved AC in baboons in 1994. Seven aseptically excised and cryopreserved controlled rate frozen osteoarticular allografts as well as two fresh allografts were transplanted into baboons and followed for up to five years. The AC was cryopreserved in glycerol. The baboons were examined every four to six months. The cryopreserved grafts healed earlier than the fresh grafts suggesting decreased immune response from the cryopreservation. All allografts developed degenerative changes at 3-4 years. The cartilage exhibited fibrillation with loss of cells in the outer layers suggesting a transformation of the hyaline cartilage to fibrocartilage, although no investigations were performed to verify this assumption. Further examination revealed some viable cells still present. At five years only three grafts remained intact. These had maintained the joint space but degenerative changes were noted on radiographs even though good ROM and function of the joints remained. On direct examination after sacrifice there was significant cartilage erosion with pitting and irregularity and, on microscopic examination, no cells were visible. The fresh grafts exhibited later healing and one of two healed by fibrous union. One fresh graft had an intact articular surface with many chondrocytes present. The authors concluded that freeze preservation did not adversely effect the biomechanical properties as seen by the satisfactory function and ROM in the joints but degenerative changes were eventually consistently present.

More recently there have been clinical trials using frozen human AC for transplantation with short periods of follow-up recorded. Flynn (14) in 1994 retrospectively reviewed 17 knees in 15 patients with a minimum follow-up of two years after transplanting fresh frozen osteoarticular allografts for osteonecrosis of the distal femur. The AC was preserved in 10% $Me_2SO$ and stored at -80°C. Mean follow-up was 4.2 years with 70% good to excellent results. These results were comparable to those from fresh nonfrozen grafts as shown by Gross (17), and Bayne (3), but not as good as those of Convery (8) (8/9 good to excellent at five years). Once again conclusions included that a precise fit was required to optimize the results and a stable, well aligned joint was essential. The fresh frozen allografts enhanced the ability to optimize these variables making an accurate fit possible with the large inventory of carefully measured allografts. The freezing also decreased the immune response and allowed quarantine to test for viral and bacterial infection in the donor. The disadvantage of the freezing was described as the undefined reduction in the chondrocyte viability which may adversely affect the outcome.

Clohisy (7) in 1994 evaluated the results of reconstruction with allografts preserved in 8% $Me_2SO$ for resection of musculoskeletal tumors of the proximal tibia. Sixteen grafts were used and 9/16 had good to excellent results over 2-5 years but there was a gradual decline in the results over time. Although the cartilage maintained its mechanical properties over short time periods there was an inability to maintain integrity over longer periods possibly due to poor survival of chondrocytes after freezing (47).

Over the past years use of fresh and cryopreserved osteochondral allografts has increased. Initial transplantation used fresh allografts attempting to maximize chondrocyte viability and allow these cells to maintain the cartilage matrix and prolong graft survival. Many papers have documented the success of these grafts and provided further indications for transplantation in the field of Orthopedic Surgery. Recent developments have occurred in an attempt to increase the success rate and safety of these transplantations by cryopreserving intact AC. Banking of AC would allow for precise size and joint contour congruency matching which is vital for successful transplantation. Successfully cryopreserving AC would also allow the procedure to be performed at appropriate times when the patient, surgeon, and other members of the transplantation team are optimally prepared. The storage of the cartilage would permit complete infectious disease testing to minimize the possibility of transmission of infectious diseases. These concerns have driven research for a consistently successful technique to cryopreserve intact AC while maintaining chondrocyte viability to enhance clinical outcome for patients who require transplantation for treatment of difficult joint problems.

## 1.3 MECHANISM OF CRYOPRESERVATION

Cryopreservation can allow survival of tissues for many years because thermally driven reactions slow down as temperature decreases. Growth and reproduction do not occur at temperatures less than -30°C. Some biochemical reactions may still occur at lower temperatures but many cell types will lose viability when stored at -78°C (26) likely due to residual intracellular liquid water. The lower limit for thermally driven biochemical reactions in aqueous systems is probably about -140°C, the glass transition point of water, as the solution becomes very viscous. With cryopreservation in liquid nitrogen (LN) down to -196°C there are no thermally driven reactions. The only reactions that occur at -196°C are photophysical events such as formation of free radicals and production of breaks in macromolecules. Based on background radiation of 0.1 rad/yr it would take 2000-4000 years to kill 68% of the cells frozen in LN.

Cryopreservation is not a new idea. Previous attempts to cryopreserve human tissues date back to the 1700's with attempted cryopreservation of sperm of soldiers who were to die in the many European wars. Successful cryopreservation began in the late 1940's augmented by the discovery of the protective action of glycerol by Polge (50) in 1949. In 1952 the first tissue bank, a bone bank, was started by the US Navy. Cryopreserved sperm had been successfully used for breeding bovines and in 1953 the first human baby was born from cryopreserved sperm. In 1984 the first human baby was born from a cryopreserved embryo and in recent years there has been increased interest in cryopreserving other human cells and tissues.

Extensive investigation into the mechanism of action of freeze preservation is ongoing but the details are not yet fully elucidated. Many events occur as cells or tissues are frozen and the specifics of these events may hold the answer to the optimal method of cryopreservation. Cells in suspension, when placed in a cooling environment, have to respond to the varying environmental conditions. As the constitution of the surrounding environment changes so must the internal mechanisms of the cells. Areas of concern in attempts to cryopreserve cells and tissues include the formation of extracellular ice, intracellular ice, increasing concentrations of electrolytes, solutes, and cryoprotectants, and damage to cell membranes via osmotic or electrical potentials.

Important variables include cell permeability to water and solutes as well as cell size and volume. There are different optimal cooling rates for different cell lines based on these factors (27). Cells with higher permeability to water allow water to escape more easily decreasing the likelihood of intracellular freezing unless the cooling rate is very fast. Cells with small volume to surface area ratios also will allow water to leave more easily and will be relatively more resistant to intracellular freezing.

A general overview of freezing of cells in solution proceeds with the initial cooling of the solution. As the solution cools past its freezing point, ice will begin to form extracellularly. Ice does not form intracellularly initially due to lack of effective intracellular nucleators (27) but water inside the cell begins to supercool. As ice forms extracellularly there is increased concentration of electrolytes as well as a difference in chemical potential inside and outside the cell causing an efflux of water from the cell to equilibrate the system. As water leaves, the cell dehydrates and the concentration of solutes inside the cell increases. If a penetrating cryoprotectant is in solution then the same events will occur but at a relatively lower temperature due to a depression of the freezing point and an increase in the nonelectrolyte solute concentration. As the temperature goes below -10°C - -15°C without cryoprotectant, the water inside the cell will eventually freeze to equilibrate the system.

Cooling of the extracellular environment below the freezing temperature of the solution does not necessarily mean the internal constitution of the cell must freeze. At temperatures warmer than the homogenous nucleation temperature there must be a nucleator present to form ice. Ice itself is a very good nucleator for surrounding water but if the cell membrane acts as a barrier between the ice and the intracellular water, ice will not be able to nucleate the water and the cell will remain unfrozen. Thus cells can supercool to temperatures of -10°C - -15°C even when there is ice in the extracellular environment (27) indicating the cell membrane can prevent the growth of external ice into the supercooled interior and that cells do not contain effective nucleators of supercooled water. Therefore, at temperatures warmer than -5°C, everything is unfrozen because of supercooling and the depression of the freezing point by the presence of protective solutes. At temperatures between -5°C and -15°C the external medium will begin to freeze but the cell will remain completely unfrozen due to the plasma membrane. If intracellular freezing occurs at temperatures less than the nucleating temperature a significant event must have occurred inside the cell.

The majority of cell injury is thought to be caused by two events. The first is "solution effects" most evident with slow freezing. The mechanism of damage due to solution effects is unknown but there are many theories (2,24,42,43). Ice formation initiates solution effects by removing water from the system as ice resulting in concentration of high and low molecular weight solutes. Cell volume decreases as a result of water loss and solutes may precipitate out. The minimum cell volume theory (41,42,67) proposed that water loss and volume reduction were responsible for the hypertonic injury associated with freezing. Certain cells were unable to shrink below 55% of normal volumes which caused the membrane to become leaky and allowed solutes to move into the cell with freezing. Upon thawing there was an increase in intracellular water and if the increase in cell solutes was too great the cell would undergo osmotic lysis. According to this theory, slow thawing should reduce cell injury by allowing an equilibration of solutes out of the cells. Subsequent studies (38) have shown increased damage to cells with slow thawing disputing this theory. Another theory (18) suggested excessive dehydration may destroy the relationship between intracellular water with surface proteins, destabilizing them and subsequently lead to protein denaturation. Unfortunately no consistent relationship is seen between cell injury and volume reduction. These events may occur at the same time and it is difficult to determine which is the main cause of damage.

It is possible that increased electrolyte concentration extracellularly may cause direct damage to the cellular plasma membranes (23). The increase in intracellular electrolyte concentration due to water efflux from the cell may directly damage intracellular organelles. Damage may also be due to flow of water out of the cell (42). With a strong osmotic or electrical gradient, water efflux may be so rapid that the membrane is irreversibly damaged. Another possibility is physical damage to cells which may occur due to the ever decreasing amount of space due to formation of increasing amounts of ice.

The second commonly thought of injurious event is formation of intracellular ice (21) usually during rapid freezing, but also during slow freezing, while the cell is still hydrated. Intracellular ice may have a solution effect inside the cell by concentrating the solutes and damaging organelle membranes intracellularly or the ice may physically damage the cellular contents when it forms. While extracellular ice is compatible with survival after freezing, intracellular ice is almost universally associated with cell death whether due to ice formation, recrystallization, or melting.

Theories on intracellular freezing that occurs above the homogenous nucleating temperature include Levitt's (22) proposal that the cytoplasm spontaneously nucleates at a critical degree of undercooling which is due to the difference in intracellular and extracellular osmotic pressures. Mazur (29,30,31) proposed that extracellular ice crystals nucleate the cell. With an increased rate of cooling the radius of the growing tip of the ice crystal decreases and the smaller tips may propagate through the plasma membrane. Another theory (59,69) stated that electrical currents exist at the growing ice interface in aqueous solution and this may contribute to the disruption of the cell membrane.

Recently Muldrew et. al. (50) examined the mechanism of intracellular ice formation. Data obtained refuted the previously quoted studies and a new theory was put forth. Muldrew et. al. showed that the plasma membrane was damaged at a critical gradient in osmotic pressure across the cell membrane and intracellular freezing occurred at this point. Once the cell was frozen, its membrane lost the ability to act as a barrier to extracellular ice allowing the ice to nucleate the cytoplasm. This mechanism worked even without the formation of ice as long as a similar osmotic gradient was reached. The authors hypothesized the cell damage could be due to the flux of water, driven by osmotic pressure, through the cell membrane.

Based on theories of events of cell freezing and the proposed mechanisms of cell injury different techniques of cryopreservation have evolved. The rate of freezing is very important. One consistent finding is that formation of intracellular ice leads to cell death. A reduction in intracellular water may prevent the formation of intracellular ice. Slow freezing may promote cellular dehydration and if cooled slowly enough, the cell would have time to equilibrate and prevent intracellular supercooling as well as maintain the intracellular chemical potential equal to that of the extracellular environment. If cells are extensively supercooled but well hydrated they are very likely to freeze intracellularly as they reach their nucleation temperature. If 90% of the free unbound water has been removed there would be no intracellular freezing (57,68) at any temperature.

Disadvantages of slow freezing are the "solution effects" mentioned earlier which may be worse with prolonged exposure (37). Prolonged exposure to the high concentration of solutes extracellularly renders the cells susceptible to solute leakage especially in the presence of additional stressors such as cooling (thermal shock) and thawing (dilutional shock) (13).

The alternative to slow cooling is rapid cooling. The main advantage of rapid cooling is less exposure to high concentration of solutes that occurs during freezing. If the solution effect is a major contributor to cellular damage, then rapid cooling may be beneficial. The problem with rapid cooling is that cells are not able to dehydrate quickly enough to equilibrate. To compensate, the cell will become increasingly supercooled and eventually equilibrate by freezing intracellularly. This severely limits rapid freezing from being used in isolation.

After freezing down to -196°C the cells must eventually be rewarmed to room temperature. The rate of warming also has a bearing on survival of cells (38). Small ice crystals tend to reduce their surface energies by forming larger ice crystals by growing or fusing together. When warming is slow there is increased opportunity for small ice crystals to form into larger ice crystals. This is especially relevant with rapid cooling as this preferentially creates smaller ice crystals (27). Mazur (28) stated that with rapid cooling survival was higher with rapid warming because there was less opportunity to recrystallize. If cooling rates were slow then slower warming rates had a higher recovery because less intracellular water was present during thawing. In a subsequent paper Farrant and McGann (38) also investigated the effect of slower warming rates on survival. No effect on survival, regardless of the warming rate, was found if the cells were frozen using a rapid cooling technique, but overall results were better with the rapid warming rate.

Mazur (27,28) noted optimal cooling rates for different cell lines to balance the effects of slow and rapid cooling. This indicated at least two factors affecting survival oppositely dependent on cooling rate supporting the hypothesis that damaging effects occur with slow cooling, solution effects, and damaging effects also occur with rapid cooling, intracellular ice formation effects. Unfortunately optimal cooling rates for most nucleated mammalian cells have not been determined. Most freezing techniques presently use some kind of controlled slow cooling rate from the freezing point to about -50°C as first promoted by Smith and Polge (50) followed by rapid freezing. McGann and Farrant (37) subsequently devised a 2-step freezing technique to control for the difficulties in freezing mammalian cellular systems. In the initial phase, cooling is performed rapidly by placing the solution at a relatively high subzero temperature. The solution is held at this intermediate holding temperature to allow the cells to equilibrate. This phase allows the extracellular solution to freeze, dehydrating the cell by osmotic and chemical effects. Once dehydrated, the cells are plunged into LN in the second step. Since minimal water remains in the cell there will be no intracellular ice formed by plunging into LN. This would effectively store the cells indefinitely. Rapid warming was used to bring the solutions back to room temperature.

McGann and Farrant concluded that damage was attributable to each step. In the first step, with long periods at the intermediate holding temperature, damage may have occurred due to thermal shock, high osmolality, or direct toxic effect of high ionic strength conditions. Protection from subsequent damage due to plunging into the LN was acquired with longer periods at the holding temperature. There may be a time dependent interaction of the hypertonic extracellular environment on the cell membrane and this may be shrinkage. The authors found this 2-step technique achieved greater cell survival compared to the constant cooling rate but also reinforced the theory of damage due to both slow freezing and rapid freezing.

The multiple uncertain mechanisms of injury have thus far prevented development of a consistently successful protocol for cryopreserving human tissues. Some general principles have been developed but more work is required to clarify these issues.

## 1.4 CRYOPRESERVATION OF ARTICULAR CARTILAGE

Articular cartilage is the most important tissue required for joint reconstruction, therefore preservation of integrity and function during cryopreservation are vital to the success of transplantation. Articular cartilage is a complex structure consisting of proteoglycans for water retention and collagen for strength. The water content of AC varies from 65-80% (48) depending on the area measured. High water content may allow AC to adhere to the same principles as individual cells in suspension during cryopreservation procedures. Previous studies (35,52,65) demonstrated that isolated chondrocytes responded in a similar manner to other cells during cryopreservation and that chondrocytes were permeable to penetrating cryoprotectants such as $Me_2SO$(36,62). Tavakol (62) elegantly showed the protective effects of 7.5% $Me_2SO$ on rabbit AC by doing a comparative study of electron microscopic visualization of chondrocytes and cartilage matrix before and after cryopreservation. Results showed that treatment with $Me_2SO$ prior to cryopreservation was associated with partial preservation of cellular structures.

Early work on AC cryopreservation was performed by Smith (55) in 1965 isolating chondrocytes from AC matrix of rabbits, dogs, monkeys, and humans using papain. Fresh chondrocytes were compared to cells cryopreserved in 10% $Me_2SO$ and frozen down to -79°C. There was a high percentage of normal appearing chondrocytes under phase contrast and fluorescent microscopic evaluation. Viability was tested by homografting the cells with successful reformation of cartilagenous-like matrix. The success of this initial work led to more extensive work. Tomford et. al. (66) in 1984 demonstrated successful cryopreservation of isolated chondrocytes after dissolving the matrix with collagenase. 60-70% cell viability after cryopreservation using $Me_2SO$ or glycerol was documented by dye exclusion assay and chromatographic analysis of cell product in culture before and after freeze-thaw manipulations.

Once isolated chondrocytes were cryopreserved, the progression was to cryopreserve intact AC. An important factor in cryopreservation of tissue is the ability of cryoprotectant to diffuse into the tissue. Permeability of isolated chondrocytes to $Me_2SO$ was investigated by McGann et. al. (36) and Schachar et. al. (53) demonstrating that 1M $Me_2SO$ required about two minutes to diffuse at 20°C. More recently in 1993, McGann et. al. (33) investigated the permeability properties of chondrocytes and the diffusion of cryoprotectant in matrix with the same results. Tomford (41) and Schachar (53) investigated optimal concentrations of glycerol and/or $Me_2SO$ useful for cryopreservation. Tomford concluded glycerol's protection was optimal at concentrations of 8-12% and $Me_2SO$ was optimal at 8-16%. Schachar found similar results with the $Me_2SO$ concentration optimal at 5-10% and minimal damage occurred to isolated chondrocytes after exposure to $Me_2SO$ at these concentrations at 4°C for up to 100 minutes. Tomford (64) noted no toxic effects of up to 20% $Me_2SO$ after 30 minutes of exposure and a small decrease in survival with exposure of 90 minutes to 12% $Me_2SO$. With progressively longer exposure there was increased loss of cell viability with even lower concentrations of $Me_2SO$. Glycerol appeared to have similar toxicity's.

With respect to penetration of cryoprotectants through AC matrix, cartilage contains a preponderance of fixed negative charges due to the anionic groups of sulfate and carboxylate in the chondroitin sulfate. Maroudas et. al. (32) demonstrated permeability of human AC to electrolytes and nonelectrolytes at room temperature. Passage of cations through the negatively charged AC was somewhat faster than that of nonelectrolyte groups which, in turn, passed somewhat faster than anions provided that molecular size was similar. Stirring also had a significant effect on the permeability. Tomford (32,64) demonstrated progressive penetration of $^3H$ - $Me_2SO$ through the cartilagenous matrix and within two hours cryoprotectant was present in the deepest layers. Muldrew et. al. (46) also demonstrated that within 30 min $Me_2SO$ will diffuse to a minimum of 2mm in intact pig AC.

Ultrastructural studies have shown that the cryopreservation procedure itself has no effect on structure or in vitro mechanics of cartilagenous matrix (1,19,62). Cryopreserved chondrocytes, on the other hand, exhibit distinct ultrastructural changes with cell membrane disruption, presence of large lipid droplets, lobulated nuclei with condensed chromatin and abnormally darkened and vacuolated cytoplasm (33). Pretreatment with cryoprotectant may ameliorate these cellular changes.

Muldrew et. al. (44) had moderate success cryopreserving intact sheep AC with up to 60% chondrocyte recovery using cryoprotection with 10% $Me_2SO$ as determined by vital dye staining. Recovery was noted mainly in the superficial and deep zones of the cartilage using a 2-phase freezing technique. Tomford (63) documented excellent cell recovery using vital dyes in the superficial zone after cryopreserving dog AC using a commonly practiced cryopreservation technique.

Cryopreservation of chondrocytes in AC is vital for its transplantation. Knowledge of isolated cell cryopreservation and cryoprotectant diffusion will aid in cryopreserving intact human AC.

## 1.5 CRYOPROTECTANTS

Cryoprotectants for cells can be classified as nonpenetrating and penetrating. Both types of cryoprotectants have been used with many cell lines including those of humans.

The nonpenetrating compounds include polyvinylpyrrolidone (PVP), dextran, and hydroxyethyl starch (HES) among others. Their mechanism of action is controversial (12,34,60). Farrant (12) in 1969 proposed that nonpenetrating cryoprotectants at higher concentrations may exhibit enhanced colligative properties and protect the cells by lowering the external concentration of salts. McGann (34) in 1978 suggested that nonpenetrating cryoprotectants may "squeeze out" water and dehydrate cells leaving them less susceptible to intracellular freeze injury. Cryoprotectants may alter physical properties of solution during the freezing process or work by other unknown mechanisms.

Taylor (61) in 1974 determined the relative abilities of nonpenetrating cryoprotectants versus penetrating cryoprotectants to cryopreserve Chinese hamster ovarian cells. Using solutions of PVP, $Me_2SO$, and glycerol, cells were frozen to -30°C, -80°C, and -196°C after immersing in appropriate solutions. During the same experimental series, they verified that glycerol almost fully penetrated at 37°C while minimally penetrated 2°C. This property allowed glycerol use to verify results achieved with PVP and $Me_2SO$ because glycerol at 37°C would be expected to give results similar to $Me_2SO$; glycerol at 2°C would have results similar to PVP. Results of this study revealed high concentrations of nonpenetrating cryoprotectants had a small cryoprotectant effect and could help potentiate the effect of penetrating cryoprotectants. The 10% $Me_2SO$ had significantly more cryoprotectant effect at all temperature levels tested. Verification was seen with glycerol at 37°C being similar to $Me_2SO$ at -196°C and even better at -30°C and glycerol at 2°C being similar to the PVP results. The conclusion was that for acceptable survival at -30°C for 24 hours, the cryoprotectant had to be present intracellularly and that this type of cryoprotection may offer additional protection at lower temperatures.

McGann (34) performed a similar experiment in 1978 using $Me_2SO$ and glycerol once again with hydroxyethyl starch as the nonpenetrating cryoprotectant. Only high concentrations of HES showed some cryoprotectant effect. The penetrating $Me_2SO$ and glycerol at 37°C were more effective as more cells survived freezing in LN. Once again the glycerol at different temperatures confirmed the different actions of different types of cryoprotectants and indicated penetrating cryoprotectants were more effective.

Penetrating cryoprotectants include $Me_2SO$, glycerol, and 1,2-propanediol (propylene glycol). Early work on penetrating cryoprotectants was done by Lovelock (23) in 1953. Examination of the mechanism of the protective action of glycerol against hemolysis during freezing and thawing showed glycerol had a protective effect against freezing via colligative action but not against mechanical or osmotic shock. Glycerol had to penetrate the cells and be present both inside and outside the cells to be effective. The concentration of glycerol required was dependent on salt concentration and rate of freezing and thawing. A faster cooling rate and a lower concentration of NaCl required less glycerol.

McGann (34) proposed a mechanism of action for penetrating cryoprotectants relating cell injury during freezing to the amount of intracellular ice, extracellular ice, and solute concentration surrounding the cells. The penetrating substances work by decreasing the amount of ice formed at any temperature during cooling which delays the increase in solute concentration at lower temperatures. This allows the cells to reach low temperatures required to afford the cells protection against further cooling. For maximum protection $Me_2SO$ must be both inside and outside the cell (36). Addition of penetrating cryoprotectant causes an efflux of water out of the cell due to the osmotic gradient. Once the osmotic gradient equalizes, $Me_2SO$ will migrate into the cell to equilibrate, soon followed by return of water back into the cell. The magnitude and rate of these changes depends on the membrane permeability to water and $Me_2SO$. These variables are strongly dependent on temperature and therefore volume changes will depend on temperature as well. These principles should be similar with all penetrating cryoprotectants but variability between substances will depend on their individual interactions with water and the cell membrane.

$Me_2SO$ and glycerol are the most commonly used cryoprotectants for human tissues and 1,2-propanediol has been used to cryopreserve red blood cells (39). Although $Me_2SO$ and glycerol are used most frequently, there is no documentation of the most effective cryoprotectant as good clinical comparison studies of various cryoprotectants are not available.

1,2-propanediol has the greatest glass forming tendency of these cryoprotectants and the stability of the wholly amorphous state may be beneficial to cryopreservation and 1,2-propanediol is associated with low toxicity (52). With this information, it is surprising that 1,2-propanediol is not used more frequently in cryopreservation. Boutron (5) in 1979 studied the stability of 1,2-propanediol. 1,2-propanediol was difficult to crystallize in concentrated aqueous solutions while no crystallization was possible in any solution with concentrations greater than 68%. At concentrations less than 50% some crystallization may occur depending on the cooling rate. Using critical warming rates to determine stability (the rate above which crystallization does not have enough time to occur), 1,2-propanediol was by far the most stable when compared with $Me_2SO$, glycerol, ethylene glycol, and glycerol plus ethanol, all at equal concentrations. Only the $Me_2SO$ at 50% V/V had a lower critical warming rate but at the 45% concentration the 1,2-propanediol was already much higher which is more relevant for cryopreservation as the concentrations used are in the lower ranges. At all concentrations and cooling rates the crystallization of the 1,2-propanediol was more difficult than other solutions which was consistent with the results from the critical warming rates. This was apparent for all concentrations, even dilute ones, and this is very important in cryobiology where the lower concentrations are required to prevent toxicity. The conclusions were that 1,2-propanediol was much more stable and could advantageously replace $Me_2SO$ and glycerol in cryopreservation techniques.

Further work was done by Mehl (37,38) trying to find a solution with a higher glass forming tendency and stability by mixing the most effective solutions for complete vitrification of tissues and organs. Mixtures of 1,2-propanediol with 1,3-butanediol and 2,3-butanediol may be particularly efficient. This has certain implications for possible use for cryopreserving cells, tissues, and organs by complete vitrification in the future but, at present, 1,2-propanediol appears to have the best potential for cryopreservation.

## 1.6 CELL RECOVERY

Understanding the sequences of events of cell death is important to understand how viability tests work. Early changes are reversible as described by Malinin (24). Alterations consist of fragmentation of cell membranes as well as changes in mitochondria and endoplasmic reticulum (ER) that occur. Injury to mitochondria and ER may initiate a severe chemical reaction which may be harmful to the cell. Later in the process, irreversible changes occur. Vacuolization occurs in the nucleus followed by hyperchromatic material appearing near the nuclear membrane. Finally condensation of the nucleus (pyknosis) demonstrates death of the cell. The nucleus may fragment or lose its ability to stain with basic dyes. Irreversible cytoplasmic structural damage facilitates the destruction of cellular structure. Autolysis is a very late event of death.

Malinin (25) examined the effects of cryopreservation of human chondrocytes using the electron microscope. Normal chondrocytes contain elaborate ER and well developed Golgi complexes in a homogenous matrix. Injured chondrocytes showed many consistent changes after varying degrees of freezing, often containing dark nuclear material , likely fragmented nuclear remnants. These crenated nuclei were consistent with cell destruction and the cell membrane was not continuous (25). Other changes noted in the cytoplasm included many secretory granules, filamentous structures, and lipid droplets. These morphologic alterations were particularly prominent in the nuclei suggesting the nucleus was the primary site of cryoinjury. These observations were consistent with those of Tavakol et. al. (62) in 1993. Using rabbit AC and cryopreserving with 7.5% $Me_2SO$, chondrocytes were evaluated under light microscopy and TEM. Abnormal nuclei with lobulation and abnormally darkened cytoplasm were seen. There were partial disruption of membranes, condensation of chromatin and large lipid droplets similar to Malinin's observations.

Determination of cell recovery and viability is difficult and not one method has been proven to evaluate cells quantitatively and qualitatively while remaining a relatively simple and easily reproducible procedure. Methods include looking at tissue cultures, membrane permeability, nucleotide incorporation, enzyme spectrum, structural alterations, chemical composition, electroconductivity, and transplantation. Transplantation would likely be the most reliable determinant of the ability of cells to survive and function normally. The difficulty with this technique is the practicality of performing transplantation prior to any preliminary work. Transplantation should be considered the ultimate test of cell survival.

Preliminary testing may include the previously mentioned tests. Tissue culture is a good technique as the number of surviving cells, as well as cell behavior as organized tissues in explant cultures, can be determined. The disadvantage of this technique is that examination of organized tissues must be performed on a relatively short term basis because cells often dedifferentiate. Another problem that may arise is the cells may undergo multiplication in a relatively short period of time thereby skewing the survival results and making this difficult to quantify accurately. It has also been seen that small amounts of cells can produce substances at relatively high rates again making it difficult to determine the percentage of survival. This is also a complex technique that is more difficult to perform on intact tissue as opposed to isolated cells. The evaluation of cell contained in matrix is very difficult and removing the cells from the matrix may add variables that are difficult to control.

Metabolic incorporation is another possible method to determine viability. In AC, $^{3}$H and $^{35}$S uptake has been used to determine cell recovery (20,53). The difficulty with this technique is that injured cells often have increased metabolism relative to normal cells (48) making accurate quantification of cell recovery unreliable.

Another method of evaluation is membrane permeability. Dye exclusion tests are based on the premise that an intact membrane is necessary to maintain the life of the cell. As mentioned earlier, one consistent finding in cells with significant damage is disruption of membranes, especially the nuclear membrane. There may be a loss of selective permeability as the cell dies. This loss of selective permeability can be used to differentiate between functional and nonfunctional cells. Initial studies often used relatively inexact stains such as hematoxylin and eosin and observed the results under relatively low magnification. Although this was sufficient to visualize completely disrupted cells it was unable to detect more subtle irreversible changes. The next generation of dye exclusion tests included the use of trypan blue (4). Although more specific than H and E staining there was still a concern about the overall specificity of this stain noted by occasional dead cells able to maintain exclusion properties with apparent maintenance of cell membranes. Trypan blue can form a complex with serum albumin and this complex can be excluded from injured or dead cells as well as live cells. This problem lead to the next generation of stains, the fluorescent dyes. The addition of fluorescein diacetate (FDA) by Rotman and Papermaster (51) allowed more specific testing. Viable cells are permeable to lipid soluble nonpolar fluorogenic ester FDA. This compound is hydrolyzed inside the cell by nonspecific esterases which release free fluorescein. Fluorescein is an anionic polar compound which can not readily leave the cell therefore it accumulates inside the cell and fluoresces green under ultraviolet light. If the membrane is damaged, the fluorescein quickly leaches out and the cell will not fluoresce.

Edidin (11) subsequently added ethidium bromide (EB) to visualize damaged cells directly. EB is cationic and can only penetrate cells with damaged membranes. It binds in the nucleus with DNA and this complex fluoresces red under UV light. Dankberg (10) in 1976 combined FDA and EB to allow direct visualization of intact cells as well as dead cells with disrupted membranes. This method was repeated by Patel et. al. (49) again showing membrane damaged cells fluoresce bright red and functional cells fluoresce bright green.

The difficulty with using these stains, most notably the FDA, is time constraints. The FDA requires incubation of the cells with the stain for absorption when chondrocytes are retained in native tissues. As well there is only a short period of time (10-15 seconds) which the dye will fluoresce and then it fades making evaluation more difficult (49). Additionally the EB is a nuclear stain and therefore is concentrated in this small area whereas the FDA is a cytoplasmic stain and covers a much broader area. To address these problems a new stain was developed called Syto. Syto is a nuclear stain like the EB and therefore will concentrate with the DNA in the nucleus of the cell if cell membranes are intact. This stain fluoresces green after binding with DNA in contrast to red caused by EB. Yang et. al. (70) recently compared the Syto stain with FDA and another stain 3-(4,5-dimethylthiazil-2-ya) - 2,5-diphenyl tetrazolium bromide (MTT) with all three combined with EB. Four model tissue systems were used including cells in suspension and cells in intact AC followed by graded freezing techniques to certain subzero temperatures to induce cell injury. Results demonstrated that the nuclear stains (Syto and EB) identified individual cells very well. Fluorescent release from the cells and fading of the fluorescent intensity were much slower than the cytoplasmic stains. Syto stain had similar recoveries as other stains utilized in other assays indicating successful staining. The authors concluded that the Syto assay was the most suitable technique for quantitative assessment of cell recovery.

Although these stains appear to be very good for determining cell recovery and intactness of individual cells they still are not able to conclusively determine the health and viability of these individual cells. These stains are a good preliminary test for procedures such as cryopreservation but more definitive accounts of viability will have to come from more extensive and complicated procedures and ultimately transplantation with clinical follow-up.

## 1.7 RESEARCH INTRODUCTION

The expanding area of osteochondral allografts in Orthopedic surgery has made this area of research more vital. Extensive scientific investigation on cryopreservation techniques and cryoprotectant solutions has provided ground work for more research directed toward articular cartilage cryopreservation. Ongoing clinical work has emphasized the need to produce successful cryopreservation of human articular cartilage. The experiments performed here will advance our knowledge and understanding through many features unique to them. Most research in the past has been done on cells other than chondrocytes or on isolated chondrocytes themselves. The opportunity to perform experiments in intact human articular cartilage with *in situ* assessment of the chondrocytes has not been performed to date. New techniques using vital dye staining have allowed the evaluation of chondrocyte recovery while the cells remain *in situ* in intact cartilagenous matrix. Other techniques such as ultraviolet microscopy and scanning electron microscopy aid in documenting the results obtained. The following four studies have combined many techniques which were not previously possible.

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# CHAPTER 2

# CRYOPROTECTION OF INTACT HUMAN ARTICULAR CARTILAGE

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Running Title: Cryopreservation of human cartilage

## 2.1 ABSTRACT

Cryopreservation of human articular cartilage (AC) will enhance clinical outcomes of osteochondral allograft transplantation. The purpose of this study was to evaluated a cryopreservation technique on human AC previously established on sheep AC. Cartilage was harvested from two human donors within 12 hours of death. The cartilage was cored into 10mm osteochondral dowels and immersed in either phosphate buffered saline (PBS) solution or 1M dimethyl sulfoxide ($Me_2SO$). Fresh controls were taken from each solution. All remaining samples underwent a freezing protocol previously established. Samples were cooled to -5°C, extracellularly ice nucleated, and then cooled at 1°C/min to intermediate holding temperatures and either directly warmed in a 37°C water bath or plunged into liquid nitrogen and then warmed. 70um slices were cut from the articular surface to the bone-cartilage junction, stained with ethidium bromide and fluorescein diacetate and viewed under an ultraviolet microscope. Intact green cells and disrupted red cells were counted and results compared to fresh unfrozen controls. Results showed excellent recovery from fresh controls (94%-97%). There was markedly decreased recovery with cooling to all intermediate holding temperatures (3%-12%) and with plunging into liquid nitrogen (4%-21%). $Me_2SO$ provided some protection to cells compared to PBS alone (11.5%:6.8%). There was a significant decrease in percent chondrocyte recovery when compared to the sheep study but recovery of cells generally followed the same pattern as in the sheep study. Further studies are required to determine the optimal cryoprotectant and freezing protocol for human AC.

## 2.2 INTRODUCTION

Cryopreservation of human articular cartilage (AC) will enhance clinical outcome of osteochondral allograft transplantation. Fresh osteochondral allografts can achieve acceptable results (5,6,13) but continue to have significant problems, including increased risk of transmission of infectious diseases (4,39), limited opportunity to achieve optimal size and joint contour congruency matches (5,6,12,13,14,46), and transplantation soon after harvesting to optimize chondrocyte recovery which may not coincide with optimal working hours. Fresh frozen allografts (4,11,21,43) have also been used with moderate success (4,11) but results tend to deteriorate after two to five years (4). This is likely due to lack of viable chondrocytes after presently used freezing techniques (38) which precludes adequate cartilage matrix maintenance resulting in early degenerative changes. Successful cryopreservation of AC can resolve these problems. More extensive screening for infectious diseases can be performed due to longer holding periods. A wide array of grafts would be available at all times allowing for better size and joint contour congruency matches as well as enabling the surgery to be performed during usual working hours when staff more familiar with these specialized procedures are available. Successful cryopreservation would provide viable chondrocytes that can maintain the cartilagenous matrix and minimize degenerative changes. Muldrew et. al. (35) successfully cryopreserved ovine AC with up to 60% chondrocyte recovery. Reproduction of these results in human AC could potentially increase the success and longevity of frozen osteochondral transplants.

Successful cryopreservation requires documentation of successful cell recovery, viability, and function. There are many methods to determine these important factors but not one method has been proven to evaluate cells quantitatively and qualitatively while remaining a relatively simple and easily reproducible procedure. Transplantation would be the most reliable indicator of successful cryopreservation with respect to the ability to survive and function normally but is not practical without preliminary work. Some studies (17,29,51) have used incorporation of $^{35}$S and $^{3}$H incorporation to indicate proteoglycan and collagen turnover respectively. Incorporation of these markers into cartilage can be misleading because as chondrocytes are injured, metabolic activity increases (39) and it is difficult to accurately correlate increased incorporation with cell survival.

The bioactive stains fluorescein diacetate (FDA) and ethidium bromide (EB) have been used to assess cell recovery based on the ability to differentiate between intact cells and those cells with disrupted membranes. Since the plasma membrane is a primary target for cryoinjury as demonstrated by TEM (21,49), an assay for membrane integrity is an appropriate and critical measure of post-thaw recovery. Initial work with dye exclusion tests to document intact cells was done with trypan blue (2). Rotman and Papermaster (41) used FDA as a more specific dye for intact cells and Edidin (9) subsequently added EB to directly visualize damaged cells. These two stains were combined by Dankberg (7) in 1976 and again by Patel (40). Ultraviolet microscopy has enabled visualization of membrane integrity as demonstrated by these vital dyes.

Membrane disruption allows permeation of EB into the cell with resultant red fluorescence. Intact membranes exclude EB while concentrating FDA in the cytoplasm where it is hydrolyzed and fluoresces green. Although FDA and EB do not directly assess cell viability, they are effective for determining cell recovery using membrane integrity of individual cells.

It is important to develop a cryopreservation technique that has a consistently high chondrocyte recovery rate and is applicable to human AC. Various methods of cryopreserving AC have been used and in 1993 McGann et. al. (27) proposed a two phase freezing technique which consisted of cooling to -5°C followed by extracellular ice nucleation and then slow freezing at 1°C/min down to a desired intermediate holding temperature. At this temperature rapid freezing in liquid nitrogen (LN) occurred. The samples were then warmed in solution in a 37°C water bath. Theoretically the initial cooling to -5°C followed by extracellular ice nucleation would allow extracellular ice to form throughout the system without damaging the chondrocytes. Further slow freezing would enabled water to efflux from the cells in response to increasing extracellular electrolyte concentration due to increasing ice formation. At a specific intermediate holding temperature enough water would have been removed from the cell (typically >90% (45,53)) such that subsequent rapid freezing by plunging into LN would not result in intracellular ice formation with its subsequent lethal effect on the cells (18). The addition of a cryoprotectant would optimize the ability of the slow cooled cells to tolerate this freezing process by its colligative effects (20).

Muldrew et. al. (35) tested McGann's approach in 1994 using sheep AC. Osteochondral dowels were immersed in cryoprotectant solution of 10% $Me_2SO$ and cooled down to various temperatures followed by plunging into LN. Cell recovery was documented using vital dye assays and resulted in 50-60% chondrocyte recovery with slow freezing down to -40°C followed by cryopreservation in LN. Preservation of the integrity of greater than 50% of the chondrocytes potentially provides sufficient chondrocytes to maintain the cartilagenous matrix. The opportunity to relate Muldrew's results to clinical practice required replication of this technique using intact human AC. This study was designed to replicate Muldrew et. al.'s experiment with the exception that human AC was used instead of sheep AC. Extensive use of $Me_2SO$ in previous studies (4,11,27,29,35,42,44,49,50,51) with articular cartilage and other human tissues (20,31,48) supported its use in this study. The goal was to determine the optimal intermediate holding temperature for human osteochondral dowels prior to freezing in LN to achieve optimal chondrocyte recovery.

## 2.3 METHODS AND MATERIALS

Cadaveric human AC was harvested from the distal femoral condyles of two donors via the Human Organ Procurement Exchange (HOPE) program. Ethical approval was obtained through the University of Alberta Hospitals Ethics Committee. Harvesting was performed within 12 hours of death of the donor and exclusion criteria included age <15 or >45 years, systemic disease involving the knee joints, or prior trauma to the knees. The AC also had to be grossly normal in appearance.

The AC was placed into physiological solution of phosphate buffered saline (PBS) solution and the experiments were performed immediately. Eighteen osteochondral dowels (OCD's) were cored from each donor using a powered coring device cutting from the articular surface into the subchondral bone. The dowels were immersed randomly into either physiological solution of PBS or cryoprotectant solution of 1M dimethyl sulphoxide ($Me_2SO$) in physiological PBS at room temperature (22°C). The samples were left in solution for 30 min to allow penetration of cryoprotectant. At this point a sample was removed from each solution to be used as a fresh control. These two samples did not undergo any cooling or freezing. The remaining 16 samples from each donor (eight in each solution) were placed in individual testtubes with 1ml of solution which was sufficient to cover the sample. The freezing protocol established by McGann and Muldrew (27,35) was followed. All 16 OCD's were placed in an ice water bath at 0°C for 10 min followed by 5 min in an alcohol bath at -5°C. At this point the samples in solution were extracellularly ice nucleated with forceps dipped in liquid nitrogen. Five more minutes were allowed at -5°C to allow dissipation of the latent heat of fusion and slow cooling commenced at 1°C/min.

At -10°C, two samples from each solution were removed. One sample from each pair was warmed in a 37°C water bath until the ice just began to melt and then was allowed to warm slowly in solution to room temperature. The other sample of each pair was plunged into LN at -196°C for a minimum of 15 minutes, then warmed in the 37°C water bath in the same way as the first two samples were. This step was repeated at four temperature gradients (-10°C, -20°C, -30°C, and -40°C) with four samples at each temperature.

Evaluation of chondrocyte recovery was performed on each sample in the same way, including the fresh controls. Vital dye assays using ethidium bromide (EB) and fluorescein diacetate (FDA) were performed on each sample as per Muldrew et. al. (35). The 10mm OCD's were secured in a TPI Vibratome 1000 (TPI, Inc., St. Louis, Missouri) and 70um slices of AC were cut from the articular surface to the cartilage-bone junction. These slices were placed in PBS solution for 15 minutes to wash out any remaining cryoprotectant (for those initially in cryopreservatant solution) and then placed on glass slides and stained with EB and FDA. The slices were incubated in the dark for 45 min for absorption of the stains. The slides were viewed under the Zeiss fluorescent microscope (440-480nm) and the ratio of intact cells to disrupted cells was recorded and compared to fresh controls.

## 2.4 RESULTS

Two complete sets of results were obtained from two cadaveric donors. Two OCD's were removed from each donor prior to any cooling - one from each solution - and used as fresh controls. The remaining samples were divided into four groups at four different temperature levels (-10°C, -20°C, -30°C, and -40°C) and the results are shown in Table 2.1 and Figures 2.1-2.4. Group #1 remained in PBS solution and was warmed directly from the intermediate holding temperature (Figure 2.1). Group #2 was also in PBS solution but was plunged into LN after reaching the intermediate holding temperature and then warmed (Figure 2.2). Group #3 was in $Me_2SO$ solution and was warmed directly from the intermediate holding temperature (Figure 2.3) while Group #4 was in $Me_2SO$ and plunged into LN and then warmed (Figure 2.4).

The fresh controls showed excellent recovery in PBS (94%) and $Me_2SO$ (97%) and these results were used as reference values for the remainder of the samples. Recovery rates at all temperature levels were calculated together to assess the effect of the $Me_2SO$ overall. The combined recovery from $Me_2SO$ was greater than from PBS overall (11.5%:6.8%), when warmed directly from the intermediate holding temperature (13.8%:7.3%) and after plunging in LN (9.3%:5.3%). There was a large decrease in chondrocyte recovery between the fresh controls in PBS and $Me_2SO$ and the samples that underwent freezing. Even Groups #1 and #3 that did not undergo freezing in LN but were directly warmed had a precipitous decrease in chondrocyte recovery noted even at the relatively high temperature of -10°C (14%). Figures 2.1 and 2.3 demonstrated warming from the intermediate holding temperature. There was decreased cell recovery as the temperature decreased. Figures 2.2 and 2.4 demonstrated recovery rates after plunging into LN. Chondrocyte recovery was greater at lower temperatures.

## 2.5 DISCUSSION

All species vary in sometimes subtle ways as exhibited with the disappointing results from this study. The experiment was terminated after the second donor due to poor chondrocyte recovery. Fresh controls revealed excellent cell recovery in both physiological solution and cryoprotectant solution demonstrating that chondrocyte integrity was maintained during harvesting and processing procedures. The control results were consistent and established a reference for further testing. There was no difference between the samples in PBS and $Me_2SO$ indicating that 1M $Me_2SO$ was not toxic to chondrocyte integrity.

The chondrocyte recovery overall was greater from $Me_2SO$ (11.5%) when compared to PBS (6.8%). This loss was evident when warmed directly from the intermediate holding temperature (13.8%:7.3%) and after plunging into LN (9.3%:5.3%) suggesting some mild potential protective effect of the penetrating cryoprotectant. The colligative properties of $Me_2SO$ would be expected to protect cells to lower temperatures resulting in higher recovery rates as seen here. The numbers were too small to make definitive conclusions but a trend was evident. There were minor variations in recovery at the four different holding temperatures with no clearly optimal intermediate holding temperature. The overall recovery rates were lower than expected as compared to those achieved by Muldrew et. al. (35).

The vast majority of cell death in this study was seen during the initial slow freeze protocol with only 9.7% average chondrocyte recovery on warming from the intermediate holding temperatures. Most surprising was poor cell recovery from even -10°C after direct warming (14%). As cells are cooled there is decreased recovery as the final temperature decreases. A high rate of cell recovery is present until conditions become unfavorable, then a precipitous drop in cell recovery should occur at a temperature specific for individual cell types (23). Addition of cryoprotectant shifts this curve to lower temperatures by colligative properties. By decreasing the amount of ice formed at all temperature levels the cryoprotectant can decrease electrolyte concentration by maintaining more water in its liquid form thereby decreasing osmotic and mechanical stresses on the cells. Cell recovery should decrease as the intermediate temperature is lowered (28) when followed directly by warming. This was seen in Muldrew and coworkers' work (35) in sheep AC immersed in $Me_2SO$ and in our human AC immersed in $Me_2SO$ (Figure 2.2) but not seen in either study in the absence of cryoprotectant (Figure 2.1). It is possible that water interacts with cells in the cartilaginous matrix differently than to cells in solution from which cell recovery curves originated. The location of chondrocytes in the matrix in relation to the water may be such that as ice forms in the matrix it mechanically, osmotically, or by solution effects disrupts the cells. $Me_2SO$ may have a stabilizing effect on either cells or water located in the matrix thereby limiting the damage to the cells and allowing them to act more like cells in suspension.

Isolated chondrocytes have been able to withstand cooling to -10°C with high recovery (34). Cell death due to freezing is thought to be due to two types of injury (1,10,20,22,23,30,33,34). Slow freeze injury is due to increased electrolyte concentrations as ice is formed and water is removed from the system. Possible mechanisms of cellular injury include surpassing minimal cell volume allowable for cell survival (32,33,52), creation of membrane damage by rapid efflux of solutes or water from the cell (33,36), or destruction of surface proteins with eventual denaturation (15). Other possible explanations may include the unique interaction between the architecture of the cartilagenous matrix and the cells in lacunae which may result in membrane damage due to retraction from the sidewalls of the lacunae or mechanical damage from the ice itself. It is possible that any or all of these mechanisms, even at the relatively warm temperature of -10°C, may be occurring and resulting in cell death. This would indicate that, unlike cells in isolation, cells in matrix are more sensitive to these mechanisms of injury or possibly other unknown mechanisms.

The second type of cellular injury is due to rapid freeze injury. Remaining intracellular water will freeze forming ice crystals which cause lethal injury to cellular membranes and organelles (19,24,25,26,36,47,54) during rapid freezing. There was little further death seen with the rapid freezing here but this was due to relatively few cells remaining after the initial slow freeze. In this type of freezing protocol, cells must first survive the slow freeze environment prior to experiencing any rapid freezing. Only relatively few cells survived the slow freeze injury therefore it is difficult to determine effects of rapid freezing.

Muldrew's study had optimal recovery in the superficial and deep zones of the AC. This may be explained by the presence of existing vascular channels from the bone-cartilage interface prior to full development of the tidemark at skeletal maturity (37,39). At maturity a tidemark between the bone and AC develops thus eradicating any communication between subchondral bone and articular cartilage. This could explain the observation in our mature human AC that the majority of any chondrocyte recovery was seen only in the superficial zone. Another possible explanation may be ice fronts (Muldrew personal communication). As ice forms outside the sample, ice may begin to migrate through the matrix from exposed surfaces. As the ice front proceeds inwards it pushes an increasing salt concentration ahead of it which may reach lethal levels towards the center of the sample.

Explanations for the difference between sheep and human AC may relate to structure and function. There is little written about sheep AC although there is information regarding other animals (8,16,17). The matrix components are comparable between species but may not be in the same configuration or concentration. This could have a profound effect on the ability of the cryoprotectant to penetrate the matrix and exert its effect on the chondrocytes. The chondrocytes themselves or the interaction between the cells and their surrounding matrix may alter the response to the cryoprotectant. Another explanation may be in the selection of the cartilage samples. Our human samples were mature adults and of larger size compared with the sheep. The size difference may be significant as cartilage thickness is proportional to stress exerted across the joint (16). The larger the stress, the thicker the cartilage becomes which, in turn, affects penetration of the cryoprotectant. These theories are difficult to prove.

Although the gross appearance and the clinical function of cartilage is similar between humans and sheep there is a fundamental difference between the two as noted by the discrepancy in chondrocyte recovery compared to previous animal studies. The extrapolation of the good results from sheep AC to use in human osteochondral transplantation requires reproduction of similar results. To accomplish this goal the best cryoprotectant and the optimal holding temperature for human AC prior to freezing must be established. Once cryopreservation of human chondrocytes in intact articular cartilage is achieved further clinical studies can be initiated.

## 2.6 ACKNOWLEDGMENTS

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## 2.8 TABLES AND FIGURES

TABLE 2.1 Chondrocyte recovery

| Temperature | % recovery | % recovery after LN |
|---|---|---|
| PBS at -10°C | 7 | 3 |
| $Me_2SO$ at -10°C | 21 | 4 |
| PBS at -20°C | 12 | 3 |
| $Me_2SO$ at -20°C | 16 | 12 |
| PBS at -30°C | 5 | 8 |
| $Me_2SO$ at -30°C | 10 | 9 |
| PBS at -40°C | 7 | 7 |
| $Me_2SO$ at -40°C | 8 | 12 |

FIGURE 2.1: Chondrocyte recovery from PBS and directly warmed

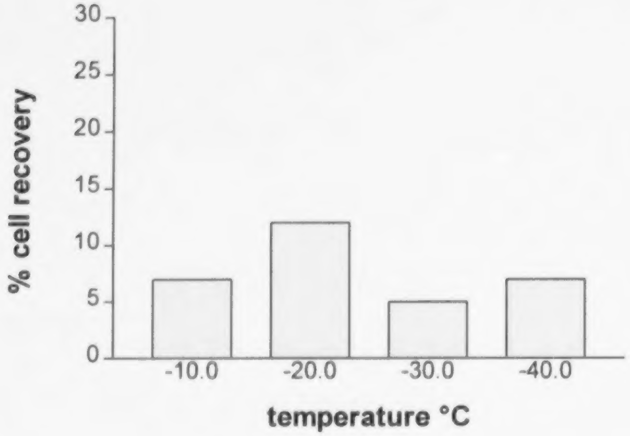
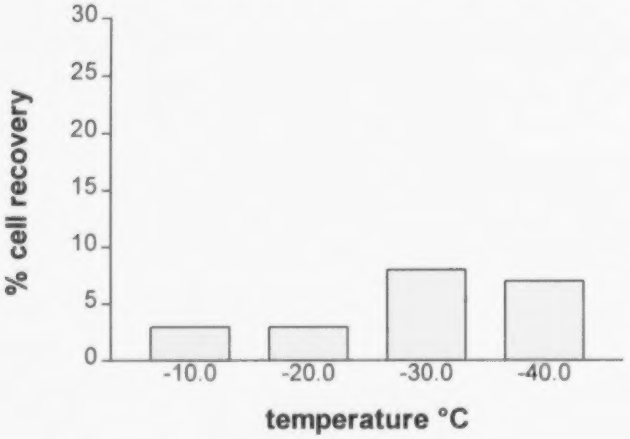


FIGURE 2.2: Chondrocyte recovery from PBS in LN



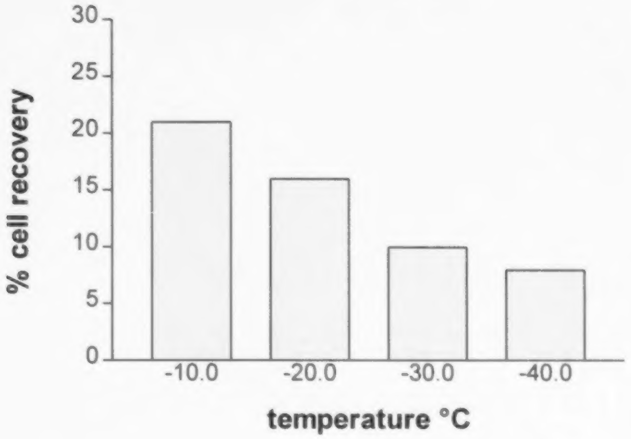


FIGURE 2.3: Chondrocyte recovery from $Me_2SO$ directly warmed

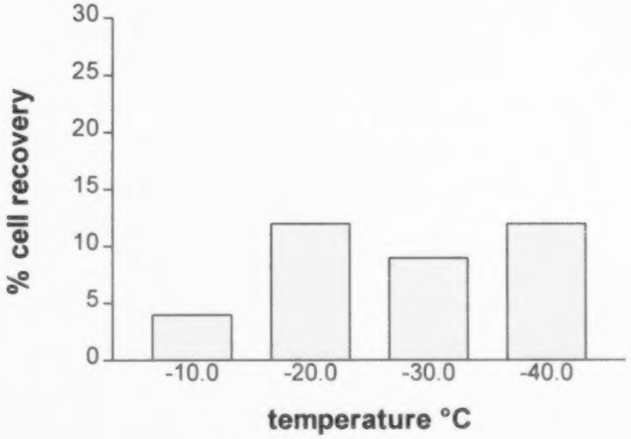


FIGURE 2.4: Chondrocyte recovery from $Me_2SO$ in LN

# CHAPTER 3

# CRYOPROTECTANT SOLUTIONS FOR INTACT HUMAN ARTICULAR CARTILAGE

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Running Title: Cryopreservation of human cartilage

## 3.1 SUMMARY

Successful cryopreservation of intact human articular cartilage has not been accomplished. The cryopreservation of articular cartilage (AC) would allow banking of this tissue aiding its transplantation in conditions such as osteoarthritis, tumors, trauma, and osteochondritis dissecans, allowing donor matching for size and shape, and enhancing screening for infectious diseases.

AC from the distal femoral condyles of four human cadaveric donors were harvested within 12 hours of death and from two patients undergoing total knee arthroplasties. 70um full-thickness slices were cut and placed into separate containers of physiological medium, 1M glycerol, 1M Me$_2$SO, 1M methanol, and 1M 1,2-propanediol. After slow cooling at 1°C/min to -30°C, samples were either thawed directly or cooled rapidly in liquid nitrogen before thawing. The samples were then stained with Syto 13 and ethidium bromide and viewed under a fluorescent microscope to quantify *in situ* chondrocyte recovery based on membrane integrity. The fresh control group had 91.7 ± 5.4% recovery (mean ± standard deviation) prior to freezing. Freezing in the absence of cryoprotectant resulted in very low recovery. There was a statistically significant increase in recovery of chondrocytes after freezing in liquid nitrogen when using the 1,2-propanediol (57.4±11.7%) when compared to glycerol (30.7±19.6%, $P<0.05$), Me$_2$SO (11.1±17.0%, $P<0.01$), methanol (1.0±0.5%, $P<0.01$), and physiological solution (8.4±11.7%, $P<0.01$). Previous studies and current clinical practice use glycerol and Me$_2$SO as cryoprotectants with human AC. This study showed that in intact human AC with a thickness of 70um, 1M 1,2-propanediol had the

highest recovery, implying that better clinical results may be achieved by using 1,2-propanediol instead of glycerol or $Me_2SO$ in the cryopreservation of intact human articular cartilage.

## 3.2 INTRODUCTION

Cryopreservation of human tissues dates back to the 1700's with attempts to cryopreserve sperm of the soldiers who were to die in the many European wars. Modern successful cryopreservation was popularized in the late 1940's with the discovery of the protective action of glycerol by Polge et. al. (33) in 1949. In more recent years there has been successful cryopreservation of individual cells such as erythrocytes (18,34), sperm (33) and chondrocytes (6,37,40) and some tissues such as embryos (12). The freezing of other tissues such as human articular cartilage (AC) should be possible but has proven difficult and elusive. Reasons to transplant articular cartilage include osteoarthritis, joint trauma, bone tumors, and osteochondritis dissecans. The ability to cryopreserve articular cartilage may enhance the technique and outcome of transplantation of articular cartilage. The past decade has seen an increased interest in articular cartilage transplantation due to the increased use of osteochondral allografts as an alternative to total joint replacement in younger patients and to repair significant cartilagenous defects such as in osteochondritis dissecans or secondary to joint resection for removal of bone tumors.

In 1985 Czitrom et. al. (9) reviewed the 14 year experience in Toronto of transplanting fresh osteochondral allografts into small osteochondral defects in over 100 patients. In the same review were 55 more patients with reconstruction of large defects due to tumor. There have been many other studies reviewing results of fresh allografts, including Garrett (15,16), Convery et. al. (8), and Stockley and Gross (38). The results of these and other papers raised important considerations regarding the results of transplantation of fresh and preserved human cartilage. The best results consistently occurred with good matches in size and joint contour congruency from donor to recipient. Other important considerations were the availability of the allografts, the opportunity to perform the surgery during elective time and the necessity to screen for disease transmission.

In response to these concerns there has been increased interest in cryopreserving articular cartilage for transplantation. Successful banking would provide a wide array of allografts to be available, allowing optimal matches in size and joint contour congruency, as well as allowing the surgery to be performed electively. Cartilage banking would also allow storage of allografts to permit more complete screening for infectious diseases.

Early work was performed by Schachar et. al. (36) on rabbit AC. Based on studies (6,36,40) showing that individual chondrocytes could maintain their viability and grow in culture following cryopreservation, survival and function of transplanted allograft cartilage was evaluated by quantitative assessment of metabolic and biochemical parameters (36). Conclusions indicated that allografts could maintain function over the one year period of study but autografts had better results overall. Malinin et. al. (19) investigated longer term results in seven baboons. Follow-up for up to five years with these small numbers concluded that although biochemical properties were not altered by the freezing technique, early degenerative changes as determined by radiographs still occurred.

More recently there have been clinical trials (7,14,21) using cryopreserved human AC for transplantation with short periods of follow-up. Flynn et. al. (14) had 70% good results in 17 patients which was comparable to Gross et. al. (17) and Bayne et. al. (2) but the results were not quite as good as those of Convery et. al. (8). Clohisy and Mankin (7) obtained results in 16 patients which showed a gradual decline in structural and functional results after two to five years. This suggested that although mechanical properties were maintained over short periods, there was an inability of the cartilage to maintain its integrity over long periods of time as assessed by the rating system of Mankin et. al. (22) and by physical examination. The poorer results may have been due to an inability to effectively cryopreserve the chondrocytes, which are required for prolonged maintenance of the cartilage matrix.

Chondrocytes maintain the structure and function of articular cartilage. For continued maintenance of matrix integrity, chondrocytes must be viable. Preservation of viability in chondrocytes during cryopreservation requires the use of a cryoprotectant. Cryoprotectants can be classified into nonpenetrating and penetrating. At this time most human cells are preserved in the presence of penetrating substances such as glycerol, dimethyl sulphoxide ($Me_2SO$), 1,2-propanediol (commonly called propylene glycol), and methanol among others. Although glycerol and $Me_2SO$ are the most frequently used cryoprotectants for human tissues there is no apparent documentation of the most effective cryoprotectant for AC.

Increasing requirements for cryopreservation of human AC has motivated this study designed to evaluate the commonly used cryoprotective agents (glycerol and $Me_2SO$) and other potentially effective agents (methanol and 1,2-propanediol). This study was performed using slices of intact human articular cartilage and a controlled cooling procedure established by McGann (25) to monitor low temperature responses, and applied to intact sheep articular cartilage by Muldrew et. al. (31).

## 3.3 METHODS AND MATERIALS

Ethical approval was obtained from the University of Alberta Ethics Committee and human articular cartilage was recovered from the distal femoral condyles of four human cadaveric donors (<45 years old) within 12 hours of death as well as from grossly normal appearing cartilage from the lateral distal and posterior femoral condyles in two patients undergoing total knee replacements (TKA). The cartilage was immediately placed in normal saline at room temperature (21°C). Exclusion criteria included grossly abnormal appearing articular cartilage. The cartilage was cored into 10mm diameter osteochondral dowels (OCD) and placed into a physiological medium (phosphate buffered saline solution (PBS) or 1M basal medium eagle (BME)). Using the TPI Vibratome 1000 (TPI, Inc., St. Louis, Missouri) the cartilage from the OCDs was cut into 70um thick slices, cutting from the exposed surface towards the bone-cartilage junction. Paired test-tubes of 1ml of cryoprotectant solution (1M $Me_2SO$, 1M glycerol, 1M methanol, and 1M 1,2-propanediol) were prepared as well as a control of physiological solution. Three slices were placed into each test-tube and the cryoprotectants were allowed to penetrate for 30-40 min at 21°C.

A fresh control sample was maintained in the physiological solution and did not undergo the freezing process. This was used as a reference for *in situ* chondrocyte recovery. The remaining samples were cooled using the freezing protocol described by McGann (25) and Muldrew et. al. (31). Samples were placed into water bath at 0°C for 10 min before transfer into an alcohol bath at -5°C for 5 min to allow temperature equilibration. Ice was then nucleated in the samples using forceps cooled in liquid nitrogen (LN). After another 5 min at -5°C to allow dissipation of the latent heat of fusion, the samples were cooled at a rate of 1°C/min to a temperature of -30°C. One sample from each pair was removed and plunged into LN at -196°C. After submersion in the LN for a minimum of 15 min, samples were rapidly warmed by immersion in water at 37°C. The samples were warmed until the ice just began to melt and then they were left at room temperature to warm to room temperature. The remaining sample of each pair was not frozen in LN but warmed directly from -30°C in the 37°C water bath.

Slices of AC were then placed onto slides and stained with bioactive stains Syto 13 (Molecular Probes, Eugene, OR) and Ethidium Bromide (EB) (Sigma, St. Louis, MO). After waiting 5 min for absorption of the stains, the AC slices were viewed under the Zeiss fluorescent microscope using ultraviolet illumination (440-480nm). Representative samples were videotaped and the individual cells were counted for the uptake of the fluorescent dyes (Syto 13 staining green for intact cells and EB staining red for disrupted cells). The total number of green and red cells were recorded and statistical analysis was performed using paired T-test and one way ANOVA. Post-hoc analysis was performed using Tukey's Test. Results are expressed as the mean and standard deviation of replicate samples.

## 3.4 RESULTS

Articular cartilage from four cadaveric donors and two total joint replacement donors were used in this study. The percentage of chondrocyte recovery in control samples from the cadaveric donors averaged 95.1% with a range of 92.5% to 97.0%. The percentage of chondrocyte recovery in control samples from the TKA patients was somewhat lower, with an average of 85.1% (84.5% and 85.7%). The overall average of the six donors was $91.7 \pm 5.4\%$. The recovery of chondrocytes in each of the experimental solutions is shown in Figure 3.1 for samples warmed directly from -30°C, and in Figure 3.2 for samples cooled in liquid nitrogen (-196°C).

Statistical analysis was performed using paired T-test, one way ANOVA and Tukey's Test. There was a statistically significant decrease in the recovery of chondrocytes warmed directly from -30°C compared to the unfrozen control. This was evident with all of the cryoprotectant solutions as well as with the physiological solutions ($P<0.02$). There was a further significant decrease in recovery when the cartilage slices were subsequently plunged into the LN ($P<0.003$ for all solutions). This occurred in all the cryoprotectant solutions and was most marked in the methanol (from 72.0% to <1%) and least marked in the 1,2-propanediol (from 81.5% to 57.4%). Using the ANOVA and Tukey Tests to determine the differences between individual solutions, the $Me_2SO$ solution was noted to have no significant difference in chondrocyte recovery from the physiological solution after warming from -196°C. The methanol solution had even lower recovery than the physiological solution when warmed from -196°C but this was not statistically significant. There was a statistically significant increase in the recovery of chondrocytes when PG was used as compared to all the other cryoprotectants for samples stored in liquid nitrogen at -196°C. Using Tukey's Test the $57.4 \pm 11.7\%$ recovery from LN in 1,2-propanediol was significantly greater than the other cryoprotectant solutions to $P<0.01$ for the control, $Me_2SO$, and methanol and to $P<0.05$ for glycerol. The only other significant difference was between glycerol (30.7%) and methanol (<1%) solutions ($P<0.05$).

## 3.5 DISCUSSION

The ability to successfully cryopreserve chondrocytes in intact human articular cartilage would have far reaching effects in the area of Orthopedic Surgery. Transplantation of fresh (8,9,15,16,38) and frozen (7,14) osteochondral dowels is already being performed and effective cryopreservation could enhance the long term results of these transplants while allowing measures to decrease the risk for the transmission of infectious diseases. To enhance the cell recovery from cryopreserved AC it is important to establish the best cryopreservation technique including the most effective cryoprotectant. Although different cryoprotectants are used clinically there is scant and variable scientific evidence of their effectiveness especially with respect to human AC.

This study was designed to determine the optimal cryoprotectant for human AC using the freezing protocol established by McGann (25,26) and Muldrew et. al. (31) on sheep. The selection of cryoprotectants was based on ones presently in clinical use for a variety of tissues. Thin slices (70um) of AC were used to allow *in situ* assessment of cell recovery while avoiding anomalies due to differences in diffusion of cryoprotectants in larger osteochondral dowels.

There are many methods to determine cell recovery, viability and function but no single method has been proven to evaluate cells quantitatively and qualitatively while remaining a relatively simple and easily reproducible procedure. Transplantation would be the most reliable indicator of successful cryopreservation with respect to the ability to survive and function normally but is not practical without preliminary work. The bioactive stains Syto 13 and EB were selected on their ability to differentiate between intact cells and those cells with disrupted membranes. Since the plasma membrane is a primary target for cryoinjury (23), the assay for membrane integrity is an appropriate and critical measure of post-thaw recovery.

Initial work with dye exclusion tests to document intact cells was done with trypan blue (3). Rotman and Papermaster (35) used fluorescein diacetate (FDA) as a more specific dye for intact cells and Edidin (11) subsequently added ethidium bromide (EB) to directly visualize damaged cells. These two stains were combined by Dankberg and Persidsky (10) in 1976 and again by Patel et. al. (32). More recently, a nuclear stain called Syto 13 which binds to nuclear DNA and fluoresces green (30), has been used with EB. Disrupted cells have nuclear binding of both Syto 13 and EB but the fluorescence of EB dominates and these cells fluoresce red. Intact cells contain only Syto 13 and fluoresce green, making it possible to easily differentiate between intact and disrupted cells. Work by Yang et. al. (41) in 1995 confirmed the capability of this stain.

Although Syto 13 and EB are effective for determining cell recovery using membrane integrity of individual cells, they do not directly address cell function. Studies (19,39) utilizing TEM have demonstrated the effects of freezing on chondrocytes. Injured chondrocytes exhibited many consistent changes including fragmented nuclear remnants as well as disruption of cell membranes. This information supports the use of membrane integrity as an assay of cell recovery after cryopreservation.

The results of our experiments showed that cells in the unfrozen control have a high level of cell integrity (84.5% to 97.0%). Chondrocyte recovery from cartilage harvested from TKA patients was somewhat lower than that from cadaveric donors which was as expected from the older cells but control samples from both donor types had excellent recovery. These results demonstrated the ability to successfully recover and assess intact chondrocytes from cadaveric donors and TKA patient donors.

The controls using the physiological media were used to document the effect on chondrocyte recovery of the two freezing endpoints (to -30°C or to -

196°C). The recovery of cells after thawing from either of these endpoints was very low (<9%), indicating that chondrocytes in articular cartilage required cryoprotection for storage in the frozen state.

The decrease in the recovery of cells in all of the cryoprotectant solutions when cooled down to -30°C, when compared to the unfrozen controls, was significantly different at $P<0.05$. The highest recovery was in the presence of 1,2-propanediol (81.5%) followed by methanol (72.0%) and then $Me_2SO$, and glycerol (65.5% and 48.6% respectively) (Figure 3.1). Recovery declined further when samples were cooled to -196ºC. Once again 1,2-propanediol resulted in the highest recovery at 57.4%. This was followed by glycerol at 30.7%, $Me_2SO$ at 11.2% and methanol at 1.0% (Figure 3.2). There was a significant difference between the 1,2-propanediol and the other cryoprotectants to a $P<0.01$ for the control, methanol, and $Me_2SO$ and $P<0.05$ for glycerol using Tukey's Test for post-hoc analysis.

The differences in recovery when different cryoprotectants were used is unlikely to be due to differences in diffusion, since the thickness of the sample (70um), and the time and temperature of exposure should allow adequate permeation of the cryoprotectant. At -30°C, all the cryoprotectants tested provided significant cryoprotection when compared to the unprotected negative control. This was consistent with colligative cryoprotection described by Lovelock (18).

Further cooling to -196°C resulted in additional damage to chondrocytes, and significant differences in recovery even with cryoprotection. During this cooling step, there is additional concentration of solutes as more ice forms. The increased concentration of cryoprotectant, particularly methanol, appeared to have deleterious effects on the chondrocytes. Of the cryoprotectants used in this study, 1,2-propanediol has the highest glass forming tendency and has the most stability of its wholly amorphous state (4). Glass transformation rather than ice crystallization in the intracellular and extracellular compartments would avoid the phase separation which results in increased concentrations of solutes in the residual liquid, which is generally felt to be damaging to cells (1,13,18,23,24,27,28,29). The effectiveness of 1,2-propanediol in conferring cryoprotection to chondrocytes in intact articular cartilage infers that induction of a glassy state may be required for optimal recovery of cryopreserved articular cartilage.

## 3.6 ACKNOWLEDGMENTS

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## 3.8 TABLES AND FIGURES

**TABLE 3.1** *Tukey's Test for Chondrocyte Recovery from -30°C*

| | BME* | $Me_2SO$ * | METH* | GLYC* | PG* |
|---|---|---|---|---|---|
| BME* | NA* | **19.08** | **21.11** | **13.78** | **24.09** |
| $Me_2SO$ * | | NA | 2.04 | **5.30** | **5.01** |
| METH* | | | NA | **7.33** | 2.97 |
| GLYC* | | | | NA | **10.31** |
| PG* | | | | | NA |

* Abbreviations are as follows: BME = basal medium eagle or phosphate buffered saline solution; $Me_2SO$ = dimethyl sulphoxide; METH = methanol; GLYC = glycerol; PG = 1,2-propanediol; NA = not applicable. Numbers in boldface type are significant values ($P<0.05$).

**TABLE 3.2** *Tukey's Test for Chondrocyte Recovery from -196°C*

| | BME* | $Me_2SO$ * | METH* | GLYC* | PG* |
|---|---|---|---|---|---|
| BME* | NA* | 0.48 | 1.33 | 3.96 | **8.71** |
| $Me_2SO$ * | | NA | 1.82 | 3.48 | **8.23** |
| METH* | | | NA | **5.29** | **10.04** |
| GLYC* | | | | NA | 4.75 |
| PG* | | | | | NA |

* Abbreviations are as follows: BME = basal medium eagle or phosphate buffered saline solution; $Me_2SO$ = dimethyl sulphoxide; METH = methanol; GLYC = glycerol; PG = 1,2-propanediol; NA = not applicable. Numbers in boldface type are significant values ($P<0.05$).

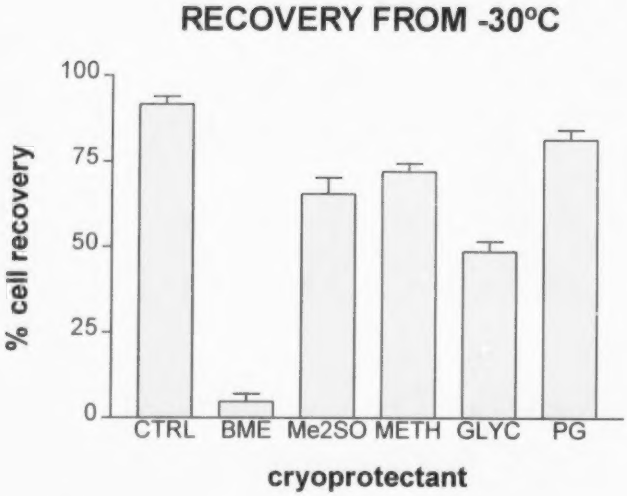

**FIGURE 3.1** Recovery of chondrocytes from freezing to -30°C. Abbreviations include CTRL = control (fresh); BME = basal medium eagle or phosphate buffered saline solution; $Me_2SO$ = dimethyl sulfoxide; METH = methanol; GLYC = glycerol; PG = 1,2-propanediol. The bars represent standard deviation.

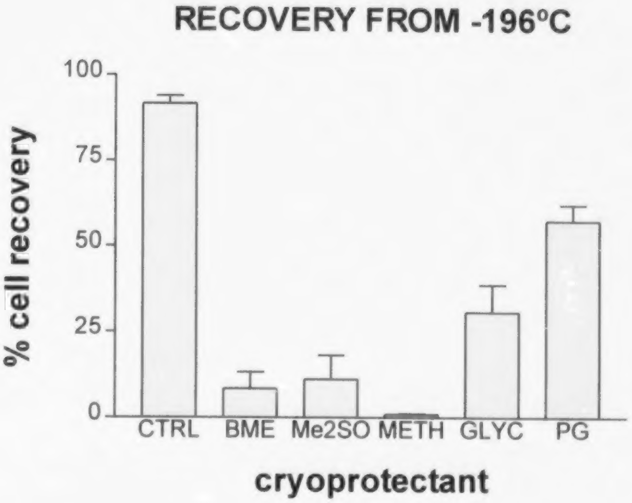

**FIGURE 3.2** Recovery from freezing to -196°C in liquid nitrogen. Abbreviations include CTRL = control (fresh); BME = basal medium eagle or phosphate buffered saline solution; $Me_2SO$ = dimethyl sulfoxide; METH = methanol; GLYC = glycerol; PG = 1,2-propanediol. The bars represent standard deviation.

# CHAPTER 4

# LOCALIZATION OF CHONDROCYTE INJURY DURING CRYOPRESERVATION OF INTACT HUMAN ARTICULAR CARTILAGE

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Running Title: Localization of chondrocyte injury

## 4.1 ABSTRACT

Successful cryopreservation of human articular cartilage (AC) would enhance the use of AC transplantation for treatment of arthritis, bone tumors, joint trauma, and osteochondritis dissecans. This study examined *in situ* chondrocyte injury in human AC under different cooling conditions. The purpose of this study was to identify specific points of chondrocyte damage during a cryopreservation procedure. Using donor AC from grossly normal appearing portions of joint arthroplasties, osteochondral dowels (OCD) were harvested and separated into four groups. Half of the samples of each group were placed into physiological solution of phosphate buffered saline (PBS) and half in cryoprotectant solution of 1M 1,2-propanediol. Each group was taken through the cooling procedure to subsequent steps (to a final low temperature of -5°C with extracellular ice nucleation) and then warmed. Chondrocyte recovery was recorded using bioactive stains Syto 13 and ethidium bromide and results relative to fresh controls samples taken prior to freezing were recorded using an ultraviolet microscope. Statistical analysis was performed using two-way ANOVA and one-way ANOVA with post-hoc analysis by Tukey's test. Results revealed no statistically significant difference when physiological solution was compared to cryoprotectant solution which indicated the 1,2-propanediol was not toxic to the chondrocytes but also that it did not provide protection to the cells and it may not penetrate the cartilagenous matrix. There was a statistically significant decrease ($P<0.05$) in chondrocyte recovery with the events of extracellular ice nucleation and with holding of the OCD's for 60 min at -5°C after

ice nucleation. This experiment identified two points of lethal chondrocyte injury during a cryopreservation procedure and will enable directed investigation to the solution of these problems with subsequent enhancement of cryopreservation of human articular cartilage.

## 4.2 INTRODUCTION

Cryopreservation of various isolated human cells has been successfully carried out for many years on cells such as sperm (4,34) and erythrocytes (25) as well as isolated chondrocytes (39,44). Since the discovery of the protective action of glycerol in 1949 (34) several different compounds which serve as cryoprotectants have been found and progress has been made improving methods for freezing the cells (18,19,20,23,24,37,38). The routine cryopreservation of human tissues has not been as successful as that for isolated cells (39,44), however, in recent years there has been increased interest in extending the techniques used to cryopreserve isolated chondrocytes to the cryopreservation of intact human articular cartilage (AC).

Successful cryopreservation of intact human AC will have significant clinical benefit in the area of Orthopedic surgery. The ability to successfully cryopreserve AC while maintaining chondrocyte viability will enhance opportunities to successfully transplant AC for treatment of common diseases such as osteoarthritis, bone tumors, traumatic joint injuries, and osteochondritis dissecans. Factors important for successful transplantation (6,10,11,41) include good size and joint contour congruency matches, ability to perform the surgery electively, a wide array of tissues to select from, and thoroughly performed infectious disease testing (3).

The ability to successfully cryopreserve human AC while maintaining chondrocyte function will satisfy all of these needs. Cartilage banking will allow a wide array of tissue to be available so the graft can be accurately matched for size and joint contour congruency. The banked tissue will be available at all times permitting the surgery to be performed during elective hours when staff more familiar with the specialized procedures are available. Banking will also permit storage of the tissue until appropriate infectious disease testing has been completed to minimize or eliminate the transmission of disease.

McGann and Muldrew (21,29) found moderate success cryopreserving intact ovine articular cartilage and noted variable results with up to 60% chondrocyte recovery when assessed using bioactive staining techniques. Work in our lab using the same cryopreservation technique but on human cartilage did not achieve similar chondrocyte recovery. This study was performed to determine the points during the cryopreservation procedure where the damage to the chondrocytes occurred.

## 4.3 METHODS AND MATERIALS

Grossly normal appearing articular cartilage was harvested during joint arthroplasty surgery and was placed immediately into normal saline. The cartilage was cut into 10mm diameter osteochondral dowels (OCD's) from the cartilage surface into bone using a hand held powered coring device and the samples were placed into either 1mL of physiological medium of phosphate buffered saline (PBS) solution or into 1mL of cryopreservation solution of 1M 1,2-propanediol (propylene glycol) in physiological PBS and allowed to saturate for 30 min. Fifty-six OCD's with cartilage 2-5mm thick were randomized into 1 of 4 groups. Group #1 was cooled for 10 min in an ice water bath (0°C) followed by 5 min in an alcohol bath at -5°C. The dowels were then warmed to room temperature in physiological solution. Group #2 underwent the same cooling procedure but after 5 min at -5°C, ice nucleation of the solution containing the OCD was initiated using forceps dipped into liquid nitrogen (LN). The container remained at this temperature for 5 more minutes for the dissipation of the latent heat of fusion and then was warmed up in a 37°C water bath until the ice just began to melt upon which the sample was removed from the water bath and warmed to room temperature (22°C) in its solution. Group #3 underwent the same treatment as Group #2 with the only difference being that the OCD's were warmed until the ice completely melted in the 37°C water bath to ensure more rapid warming to room temperature. Group #4 was cooled and nucleated in the same manner as Groups #2 and #3 but the OCDs were held for 60 min (compared with 5 min at -5°C) prior to warming in the 37°C water bath until the ice just began to melt. Once again the samples were then warmed to room temperature.

Prior to undergoing the different treatments all the OCD's had 70um slices removed using the TPI Vibratome 1000 (TPI, Inc., St. Louis, Missouri) by cutting from the articular surface towards the bone-cartilage interface. The slices were placed onto glass slides and stained with a mixture of Syto 13 stain (Molecular Probes, Eugene, OR) (15uL) and ethidium bromide (EB) (Sigma, St. Louis, MO) (100uL of 10uL EB in 1000uL PBS) in 985uL of PBS. After allowing 5 min for absorption of the stains the slices were viewed under the Zeiss fluorescent microscope (440-480nm) using ultraviolet illumination. The intact cells that fluoresced green with Syto stain were counted and compared to the number of cells that fluoresced red with EB. The percentage of intact cells were recorded and these were used as fresh unfrozen controls. The dowels then underwent various treatment protocols and were taken out of solution and placed into physiological solution. Once again 70 um slices were cut and these slices were held in physiological solution for a minimum of 15 min to allow for dispersion of cryoprotectant solution from the matrix of the AC. These slices were stained as before with Syto 13 and EB and the red and green cells were counted using the fluorescent microscope. The cell recovery in these slices after undergoing treatment were compared to their own fresh unfrozen controls and the relative recovery of chondrocytes were recorded.

Statistical analysis was performed using the two-way and one-way ANOVA with post hoc analysis done by Tukey's test.

## 4.4 RESULTS

Fourteen donors were utilized resulting in 14 osteochondral dowels in each of the four groups. In each group the 14 OCD's were divided into two groups resulting in seven OCD's in PBS and seven in 1M 1,2-propanediol for each of the four treatment groups. Table 4.1 shows the average recovery of intact chondrocytes for each group in each solution. The average recovery rate of the fresh controls was 64.6%

There was no statistically significant difference between the controls of any of the four treatment groups or the fresh controls. Two-way ANOVA revealed no significant difference between the physiological solution and the cryoprotectant solution in any of the four treatment groups. There was also no significant difference between the recovery of intact cells in the group with warming until the ice just began to melt (Group #2) and the group that experienced more rapid warming (Group #3).

One-way ANOVA with post hoc analysis using Tukey's test revealed there was a significant decrease ($P < 0.05$) between the recovery of chondrocytes from Groups #2 and #3 and the recovery from Group #1. The main difference in the treatment between these groups was that Groups #2 and #3 underwent nucleation prior to warming whereas Group #1 was directly warmed from the supercooled state without extracellular ice nucleation. There was a further statistically significant decrease in recovery ($P < 0.05$) when comparing Group #4 with Groups #2 and #3. The difference in treatment of these groups was that Groups #2 and #3 were held for only 5 min at -5°C after nucleation prior to warming while Group #4 was held for 60 min at -5°C after nucleation prior to warming.

## 4.5 DISCUSSION

The successful cryopreservation of articular cartilage will be an important step to enhance the clinical application of transplantation of human AC. There have been numerous studies using fresh (2,6,13,41) and frozen (5,10,17) AC with variable success. The studies using frozen AC typically show early degenerative changes within two to five years post-operatively. It is likely that these degenerative changes are due to loss of the chondrocytes during freezing which leaves the cartilage unable to maintain the structural integrity of the cartilagenous matrix which is vital for continued physiological function. Another important factor may be the difficulty in obtaining anatomic size and joint contour congruency matches due to limited donor selection, resulting in an incongruent joint and early degenerative changes.

Our previous inability to successfully realize Muldrew's results with ovine cartilage using human AC led to the present study to determine points during cryopreservation at which chondrocyte damage occurred. This study used the initial portion of the freezing technique employed in previous work by McGann and Muldrew (21,29) with ovine AC. Differences in the present study included using 1,2-propanediol instead of dimethyl sulfoxide ($Me_2SO$) as the cryoprotectant solution and Syto 13 as a bioactive stain instead of fluorescein diacetate (FDA).

We are attempting to extend information from previous research to human AC for future use in clinical osteochondral transplantation. 1,2-propanediol was selected, as opposed to $Me_2SO$, as a result of previous work in our lab with thin slices of human AC showing that 1,2-propanediol was a superior cryoprotectant using the present freezing technique (15). Syto 13 stain was used instead of FDA as we found it to be a superior alternative. The action of these two stains in combination with EB is similar. EB can be absorbed only by cells with disrupted membranes and therefore stains disrupted, or dead, cells red (7,8,33). Intact cells exclude the EB and take up either FDA or Syto 13 causing these cells to appear green under UV illumination. FDA is taken up into the cytoplasm where it is hydrolyzed (35) and fluoresces green, while the Syto 13 binds to nuclear DNA and fluoresces green as well. It is easier to see specific cells with the Syto 13 and it is more resistant to photo-bleaching. Its efficacy in staining intact cells compared to FDA has been validated by Yang et. al. (45).

The fresh control recovery rate of 64.6% was lower than previous rates in fresh cadaveric donor AC (15) and in Muldrew's study with sheep. This difference is likely due to early degenerative effects not grossly visible in the older samples. There was no statistically significant difference between the control solution of PBS and the cryoprotectant solution of 1M 1,2-propanediol with any temperatures or methods used, a result that was somewhat unexpected. Ice formation results in an increase in electrolyte concentration in the unfrozen compartment of the solution. In cell suspensions, the cells are confined to this unfrozen fraction and are thus subject to high solute concentrations. The increase in concentration may damage the cells by numerous proposed mechanisms (28) including surpassing minimal cell volume allowable for survival (26,27,40), creation of membrane damage by rapid efflux of solutes (30) or water (27) from the cell, or destruction of surface proteins with eventual denaturation (14). The architecture of AC may further expose the chondrocytes to membrane damage due to retraction from the sidewalls of the lacunae or mechanical damage from the ice itself. 1,2-propanediol reduces ice formation during freezing through colligative action, thereby reducing the stress that the cells are subject to at a given temperature. If the cryoprotectant solution is acting in intact cartilage as it does on individual cells (22,37,43) then there should be increased recovery of chondrocytes whenever ice is formed in the presence of cryoprotectant solution because the cryoprotectant will decrease the amount of ice formed and similarly decrease subsequent lethal effects.

Upon extracellular ice nucleation at -5°C, 1,2-propanediol reduced the amount of ice formed compared to a solution without cryoprotectant and was expected to reduce any damage to the cells via the solution effects mechanism (1,16,27) as well as by direct mechanical injury. The results were not consistent with this expectation, there was a significant decrease in chondrocyte recovery upon extracellular ice nucleation at -5°C in samples in PBS solution but there was an equal decrease in recovery in samples in 1,2-propanediol solution. Thus no protective effect was provided by the 1,2-propanediol in samples held for 5 min or for 60 min at -5°C. Using these same results it was apparent the there was no toxic effect exhibited by the 1,2-propanediol either. Toxicity of 1,2-propanediol would be expected over longer periods of time or with increased concentrations and there would be an expectant decrease in chondrocyte recovery with either of these two situations. This did not occur as noted by the equivalent recovery rates within all four sample groups indicating 1,2-propanediol was not toxic to the chondrocytes in these conditions.

Group #2 and Group #3 differed only in the OCD warming duration and rate. This step was to determine whether kinetics of the phase change during melting was important. Group #2 was warmed to until the ice just began to melt in the 37°C water bath and then was place in solution at room temperature to complete the warming. Group #3 was warmed in the 37°C water bath until all the ice was melted. During previous experiments it was noted the time of sample removal from the water bath was subjective and it was important to note any difference in chondrocyte recovery with removal from the water bath at different points during ice melting. There was no difference in chondrocyte recovery between these two groups indicating that the chondrocyte loss was not due to the difference in warming techniques after 0°C (i.e. when the ice began to melt).

The next important observation was the excellent recovery of cells from the -5°C in the alcohol bath without ice nucleation. Previous work in our lab demonstrated that 5 min at -5°C in the alcohol bath will cool the sample to -5°C. The 95-99% recovery rate indicated that human chondrocytes were able to withstand supercooling down to -5°C without damage to the cellular membranes. Again this was evident in physiological solution alone as well as in cryoprotectant solution. The damage to the cellular membranes did not occur during the supercooling or rewarming phases and there was no temperature sensitivity at -5°C.

There was a statistically significant decrease in chondrocyte recovery when comparing samples with ice to those that were not nucleated. Once again this occurred to the same extent with and without cryoprotectant. It was apparent that some destructive event occurred when extracellular ice formed or subsequent to this, such as when the newly formed ice melted. The highest recovery was noted in the superficial zone in this experiment. Muldrew et. al.

(29) noted recovery to be maximal in the superficial and deep zones and least in the intermediate zone in the sheep model. If ice was entering the cartilage matrix from both the bone and the cartilage surface, then an increase in electrolyte concentration would occur in the interior of the cartilage. This high salt concentration may have been lethal to the intermediate zone cells.

Although the sheep model used skeletally mature animals, the calcified zone between the cartilage and subchondral bone may not have been fully closed. At maturity a tidemark develops and effectively ends any direct communication (32) which may preclude the presence of ice in the deep layer in human AC. In mature humans this would allow recovery mainly in the superficial zone and possibly explain the poorer recovery from the deep zone as seen in this study. Any of these mechanisms may have been lethal to the cells themselves or they may have damaged the cellular membranes and allowed intracellular nucleation of the supercooled cells (9). Intracellular freezing was not expected to occur as the latent heat of fusion should have quickly brought the temperature of the freezing sample up to the melting point by the time ice reached the cartilage. Whatever the mechanism of cellular membrane damage there was a significant decrease in chondrocyte recovery after the extracellular ice nucleation. It is important to note that other cells have recovered from freezing to -5°C and lower with high recovery rates including leukocytes (7), stem cells (12), and sperm cells (4,34) indicating that human cells in general are not sensitive to freezing to -5°C. Also of note is freezing of isolated human chondrocytes (36,39,43,44) and chondrocytes in thin slices of intact human AC

(15) has been successfully performed to temperatures lower than -5°C with high recovery rates indicating that chondrocytes themselves are not uniquely sensitive to freezing to -5°C.

A further loss of chondrocytes occurred with longer exposure of the cells at -5°C following extracellular ice nucleation. The most likely explanation is that the exposure to high electrolyte concentrations caused a solution effects injury to the chondrocytes. The possibility of cryoprotectant toxicity is probably not responsible for injury on such a short time scale.

The results of this study pose some interesting questions. The loss of chondrocyte recovery at two stages (extracellular ice nucleation and prolonged exposure to a frozen environment at the relatively high temperature of -5°C) is unusual. Although the loss of recovery with increasing exposure to a high salt concentration is easily explained as solution effects injury, the injury that occurs immediately upon nucleation of ice is less amenable to explanation using accepted theories of cryoinjury. The effect is probably not due to intracellular ice formation because the latent heat of fusion raises the temperature of the extracellular medium to the melting point. There appears to be no chilling injury, as the same thermal regimen in the absence of ice leads to full recovery.

The negligible effect of the cryoprotectant is not easily explained either. Based on the ability of penetrating cryoprotectants to cross cell membranes and diffuse into the cartilage matrix, it was expected that there would be some protection afforded when ice was formed at -5°C. The lack of any significant effect argues against the hypothesis that the exposure injury was due to solution effects injury as commonly understood (i.e., with respect to isolated cells in suspension). Perhaps there is an intimate relationship between the chondrocytes and their lacunae which is not accounted for in the standard theory. An alternative is that the 1,2-propanediol was not penetrating the matrix at all and thereby affording no protection to the chondrocytes. After 30 min of exposure, there should have been adequate penetration to a depth of at least 2mm (31,42). Such partial penetration should have resulted in an increase in recovery of chondrocytes, especially at the periphery of the tissue, which was not noticed with examination of the slices nor the statistical analysis.

In conclusion there was no difference noted in warming rates after 0°C was reached. There was no protective effect of the 1,2-propanediol on the chondrocytes and no toxic effect was noted either. There was a statistically significant decrease in chondrocyte recovery with the event of extracellular ice nucleation and another decrease with prolonged exposure of the intact articular cartilage to ice at -5°C. The ability of 1,2-propanediol to penetrate the cartilagenous matrix requires further investigation as do the mechanisms of damage that occurred at the two points identified in this study. Directed investigation to the solution of these problems will enhance our understanding of the cryobiological behavior of intact articular cartilage and further us to the goal of banking osteochondral tissues for transplantation.

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Numbers include standard deviations. Abbreviations are as follows:

Group #1

PBS C 5 = 1X PBS control in ice for 5 min

PBS S 5 = 1X PBS sample in ice for 5 min

PG C 5 = 1M 1,2-propanediol control in ice for 5 min

PG S 5 = 1M 1,2-propanediol sample in ice for 5 min

Group #2

PBS C 5 Q = 1X PBS control in ice for 5 min, warmed quickly

PBS S 5 Q = 1X PBS sample in ice for 5 min, warmed quickly

PG C 5 Q = 1M 1,2-propanediol control in ice for 5 min, warmed quickly

PG S 5 Q = 1M 1,2-propanediol sample in ice for 5 min, warmed quickly

Group #3

PBS C 5 S = 1X PBS control in ice for 5 min, warmed slowly

PBS S 5 S = 1X PBS sample in ice for 5 min, warmed slowly

PG C 5 S = 1M 1,2-propanediol control in ice for 5 min. warmed slowly

PG S 5 S = 1M 1,2-propanediol control in ice for 5 min. warmed slowly

Group #4

PBS C 60 = 1X PBS control in ice for 60 min

PBS S 60 = 1X PBS sample in ice for 60 min

PG C 60 = 1M 1,2-propanediol control in ice for 60 min

PG S 60 = 1M 1,2-propanediol control in ice for 60 min

## 4.8 TABLE

TABLE 4.1 *Chondrocyte recovery from various conditions*

| Group #1 | Group #1 | | Group #1 | Group #1 | |
|---|---|---|---|---|---|
| PBS C 5 W | PBS S 5 W | % Recovery | PG C 5 W | PG S 5 W | % Recovery |
| 64.2±7.6 | 63.5±6.6 | 98.9 | 62.1±11.5 | 59.0±9.6 | 95.0 |

| Group #2 | Group #2 | | Group #2 | Group #2 | |
|---|---|---|---|---|---|
| PBS C 5 Q | PBS S 5 Q | % Recovery | PG C 5 Q | PG S 5 Q | % Recovery |
| 61.8±5.9 | 30.4±8.9 | 49.2 | 65.7±6.7 | 34.0±14.2 | 51.8 |

| Group #3 | Group #3 | | Group #3 | Group #3 | |
|---|---|---|---|---|---|
| PBS C 5 S | PBS S 5 S | % Recovery | PG C 5 S | PG S 5 S | % Recovery |
| 67.5±12.7 | 36.5±19.8 | 54.1 | 63.6±15.8 | 28.9±9.3 | 45.4 |

| Group #4 | Group #4 | | Group #4 | Group #4 | |
|---|---|---|---|---|---|
| PBS C 60 | PBS S 60 | % Recovery | PG C 60 | PG S 60 | % Recovery |
| 64.6±12.0 | 16.3±11.8 | 25.2 | 66.9±7.7 | 14.2±10.5 | 21.2 |

# CHAPTER 5

# MORPHOLOGY OF HUMAN ARTICULAR CARTILAGE WITH CRYOPROTECTION AND FREEZING

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Running Title: Morphology of freeze injury

## 5.1 ABSTRACT

Cryopreserved human articular cartilage (AC) is in increased demand due to potential use for treatment of diseases such as bone tumors, joint trauma, and osteoarthritis. The purpose of this study was to provide a baseline description of chondrocyte and cartilagenous matrix morphology during various steps in cryopreservation as determined by scanning electron microscopy. AC was harvested from grossly normal portions of joints undergoing joint arthroplasties. Osteochondral dowels were cored and immersed in solutions of phosphate buffered saline (PBS), 1M 1,2-propanediol (PG), or 1M dimethyl sulfoxide ($Me_2SO$). Fresh controls were fixed with glutaraldehyde and osmium tetroxide. Other samples underwent various freezing protocols followed by freeze substitution and fixation with osmium tetroxide. 70um slices were cut from articular surface to subchondral bone and scanning electron microscopy was performed. Fresh controls exhibited homogenous matrix and intact cells with no gross abnormalities. Cells in cryoprotectant solutions consistently showed shrinkage with retraction from sidewalls of the lacunae. The contracted appearance of the cells in the presence of cryoprotectant indicated that cells *in situ* did not react similar to cells in suspension. The process of cooling to -5°C resulted in mild shrinkage of cells with excess perilacunar water and no protection from intracellular ice formation with rapid freezing. The process of cooling to -30°C resulted in extreme contraction of cell volume with minimal intracellular ice and excess collections of water in the matrix. Although AC is 65-80% water, the chondrocytes maintained in its matrix do not respond like cells in

suspension. Further research is required to explore the differences in response to cryoprotectant agents and freezing conditions.

## 5.2 INTRODUCTION

Demand for cryopreserved human articular cartilage is increasing due to its potential for treatment of diseases such as bone tumors, joint trauma, and osteoarthritis. Present use of intact articular cartilage is fresh or fresh frozen with problems of availability (4,8,9,39), disease transmission (3,43), and viable chondrocyte recovery (8,14,36). Successful cryopreservation would solve these problems and allow banking of large amounts of AC for use in Orthopedic surgery.

Work in cryopreserving articular cartilage began in 1949 (31) with the discovery of the protective action of glycerol. Research was initiated by Smith (37) and evolved over the years with work by Tomford (43,44,45), Schachar (33,34,35,36), McGann (19,20,21,22), Muldrew (26,27,28) and others. Research on chondrocytes has used dimethyl sulfoxide ($Me_2SO$) (18,22,35,44,45), glycerol (35,44,45), and 1,2-propanediol (propylene glycol) (11), penetrating cryoprotectants whose action is mainly due to colligative effects (13) provided by the penetration of the cryoprotectant into the cells and the subsequent alteration of physical and chemical properties (20,22). The mechanism of action of nonpenetrating cryoprotectants is not completely understood. Proposed mechanisms include enhanced colligative properties with cellular protection by lowering the external salt concentration (7) or by dehydration (20,41).

The majority of work with AC has been done with glycerol and $Me_2SO$ although recently some work has been performed with 1,2-propanediol. These penetrating cryoprotectants are expected to work by equilibrating into the cells and lessening the effect of ice formation at all temperature levels where ice is formed. The presence of the cryoprotectant decreases the electrolyte concentration when ice forms thereby protecting the cells from the "solution effect" (1,13,25). This allows cells to survive to lower temperatures to enable water to leave the cell in response to increasing electrolyte concentrations thereby decreasing the extent of lethal intracellular ice formation (12).

The effects of penetrating cryoprotectants are proven in cells (13,23,42) other than chondrocytes in intact articular cartilage matrix. Previous work (publication pending) has provided results suggesting that penetrating cryoprotectants of $Me_2SO$ and 1,2-propanediol do not supply the protection in intact human articular cartilage that they do in individual chondrocytes. These results have introduced doubt in the ability of penetrating cryoprotectants to penetrate and protect intact human AC as was demonstrated by Muldrew et. al. (28) on pigs.

The goal of this study was to provide a baseline description of chondrocyte and cartilagenous matrix morphology during various steps in cryopreservation as determined by scanning electron microscopy (SEM). Further samples were utilized to prove or refute some commonly accepted cryobiological principles as they applied to intact human articular cartilage.

## 5.3 METHODS AND MATERIALS

Ethical approval was obtained from the University of Alberta Ethics Committee. Intact articular cartilage was harvested from grossly normal appearing articular cartilage during joint arthroplasties. These were immediately placed in sterile saline and processed. A powered coring device was used to core 10mm full thickness osteochondral dowels proceeding from the articular surface into the subchondral bone. The samples were immersed into one of three solution - phosphate buffered saline solution (PBS), 1M 1,2-propanediol (propylene glycol - PG), or 1M dimethyl sulfoxide ($Me_2SO$) for 30 min at room temperature (22°C).

Two samples were used as fresh controls, one in PBS solution and one in PG solution. These samples did not undergo freeze substitution but underwent direct fixation from room temperature. These samples were submersed in 10% glutaraldehyde for two days. The samples were transferred into osmium tetroxide for two days followed by 100% methanol. They were subsequently placed into 100% ethanol for final preparation for the scanning electron microscope.

The remaining samples underwent freeze substitution followed by scanning electron microscopy. One sample was used as a negative control and placed initially in PBS solution, then plunged directly into LN from room temperature. Two samples were placed into PBS solution followed by placement of the samples in solution in an ice-water bath (0°C) for 10 min, then a -5°C alcohol bath for 5 min. These samples were extracellularly ice nucleated using forceps dipped into LN and held for 5 min or 60 min for dissipation of latent heat of fusion. These samples were plunged into LN and then underwent freeze substitution. Two more samples experienced the same freezing protocol as just mentioned with the exception that they were initially placed into PG for 30 min instead of PBS.

Three further samples were placed in either PBS solution, PG solution, or $Me_2SO$ solution for 30 min. These samples were left in solution and placed in the 0°C water bath for 10 min, -5°C alcohol bath for 5 min, then allowed 5 more minutes for dissipation of the latent heat of fusion. These samples were then cooled at 1°C/min to -30°C, plunged into LN and freeze substituted.

The freeze substitution protocol consisted of manually removing the majority of extracellular ice from the sample and placing the sample in 5mL methanol at -60°C which completely covered the sample. The methanol was changed at 1-2 day intervals as the displaced ice diluted the methanol. After the ice had completely melted (typically seven days) the sample was removed from the methanol and placed into osmium tetroxide at -60°C for two days. The samples were removed from the freezer and placed, in solution, into a container with 100% methanol at -40°C suspended over dry ice contained in a styrofoam box. Over the next few days the dry ice was allowed to melt thus allowing the methanol and the sample to warm up. The sample was removed from the methanol bath once the temperature reached 0°C - -10°C. The sample was removed from the Osmium and placed into 100% methanol. The 10mm osteochondral dowel was placed into a TPI Vibratome 1000 (TPI, Inc., St. Louis, Missouri) and 70um slices were cut from the articular surface to the bone-cartilage junction. These slices were placed back into the methanol and transferred into 100% ethanol and prepared for the scanning electron microscope.

Scanning electron microscopy was perform with three to four 70um slices for each sample. Micrographs were taken of representative areas from each sample.

## 5.4 RESULTS AND DISCUSSION

Ten osteochondral dowels were harvested from five donors during joint arthroplasties. Representative micrographs are seen in Figures 5.1 - 5.10. Two positive controls - one in PBS solution and one in 1,2-propanediol were used. These samples were fixed in glutaraldehyde to preserve the matrix and chondrocytes from their appearance at room temperature immediately after harvesting. Our experience and fresh controls from these samples have demonstrated >90% intact chondrocyte recovery using vital dye assays (2,5,6,11,29,32,49). These fresh controls were used as references for the procedures done on the remaining samples.

Figure 5.1 is a fresh control in PBS solution. The cells completely filled the lacunae without any breaks in sidewall contact. There was no visible intracellular disruption or regions indicative of ice formation consistent with previous vital dye assays (11). The matrix appeared homogenous without distortion or fragmentation. This appearance was consistent throughout all slices viewed.

Figure 5.2 shows a fresh control in PG. Exposure to PG for 30 min at 22°C resulted in no evident disruption of the matrix with a homogenous appearance prevailing except immediately adjacent to the lacunae. Unlike the matrix in the fresh PBS control there were areas of coarse fiber orientation present. Penetration of cryoprotectant into the matrix would initiate osmotic shrinkage in cells. Subsequently as the penetrating cryoprotectant penetrated the cell there would be a concomitant influx of water following the concentration gradient back into the cell. Once cryoprotectant and water had equilibrated, the cell should have returned approximately to its original size. Based on calculations regarding solute permeation (28) significant penetration of PG into the matrix would be expected after 30 min at 22°C. Even a small amount of penetration would be evident on the cells in the periphery of the slices. The cells appeared intact without any intracellular disruption but did not fill the lacunae completely, often with sidewall attachments visible but also sometimes without. This was consistent throughout all the slices and throughout the full thickness of each slice regardless of the proximity to the edge of the cartilage.

Figure 5.1 shows the PBS fresh control as expected. There was no change in cell appearance or matrix morphology. Figure 5.2 shows the fresh control in PG solution with cells that remained shrunken, and this was uniform across the thickness of the cartilage. These two different appearances correlate with equivalent chondrocyte recovery rates as determined by vital dye assays

(11). This implies either a) the cryoprotectant was diffusing into the cartilage, equilibrating in the extracellular space, but not permeating the cells, hence resulting in water efflux from the cell, or b) there was a barrier to diffusion of PG in adult human AC, resulting in water diffusion out from the matrix and osmotic cell shrinkage.

Figure 5.3 shows a negative control of PBS solution plunged directly into LN from room temperature. The matrix consisted of a homogenous if not somewhat grainy appearance but with no specific areas of increased porosity around the lacunae. This was due to the water present within the matrix without excess collections around the lacunae. The cells themselves displayed marked irregularity in content. There was no obvious shrinkage in cell size noted by multiple areas of contact between the cell and the lacunar wall. The center of the cell had a blown out appearance marked with multiple holes representing extensive intracellular ice formation. This would be consistent with cryobiological theories regarding freezing of intracellular water with rapid cooling. This type of freezing treatment consistently results in virtually no chondrocyte recovery. The rapid cooling rate prior to intracellular freezing would not allow efflux of water from the chondrocytes, resulting in extensive lethal intracellular freezing.

Figures 5.4 and 5.5 represent samples initially in PBS solution and cooled to -5°C followed by extracellular ice nucleation and then freeze substitution. The purpose of this portion of the study was to determine the effect of slow cooling and introduction of extracellular ice prior to rapid freezing in LN. This treatment should result in increased extracellular electrolyte concentration by virtue of removal of water from the system in the form of ice. Increased electrolyte concentration should, in turn, result in efflux of water from the cells themselves and into the surrounding matrix with more cellular shrinkage followed by less intracellular ice formation. The relatively high temperature of -5°C may not be low enough for sufficient extraction of water from the chondrocytes to prevent intracellular ice from forming (38,47).

The matrix in these samples demonstrated some perilacunar ice formation indicating efflux of water from the cells. Spaces representing ice formation formed after the freezing procedure that were not present in any of the control samples. There was also mild shrinkage of chondrocytes exhibited by a small amount of cellular retraction from the lacunar sidewalls. Intracellularly there was excessive amounts of intracellular ice formation noted by extensive cellular perforations on SEM. These micrographs showed that although cooling down to -5°C forced some water out of the cells, it was not a sufficiently low enough temperature to dehydrate the cells adequately to prevent intracellular ice formation with subsequent lethal effects on cells. This result correlated well with our clinical experience of chondrocyte recovery using vital dye stains.

Figures 5.6 and 5.7 are samples exposed to PG, then treated similarly to Figures 5.4 and 5.5. The matrix in these samples was similar in appearance to those in Figures 5.4 and 5.5 with some disruption representing excess water from the cells in the matrix undergoing subsequent freezing. There was more perilacunar water present after addition of PG compared with PBS alone. The cells in these samples had a similar appearance to those in Figures 5.4 and 5.5

with multiples holes present indicating excessive intracellular ice formation. One difference noted was increased chondrocytes shrinkage in PG compared to PBS, similar to the fresh control samples.

These results are explicable if taken in context with the fresh controls. The fresh controls in PG are already shrunken to a certain extent and this preexisting diminution of water content was carried over into subsequent steps of the freezing protocol therefore at all stages there was less intracellular water and more perilacunar water in samples in PG than those in PBS. This was still not sufficient shrinkage to eliminate intracellular ice formation and the subsequent demise of the cells.

Figures 5.8, 5.9, and 5.10 represent samples nucleated at -5°C and cooled at 1°C/min to -30°C. The sample represented in Figure 5.8 was in PBS solution, Figure 5.9 in $Me_2SO$ solution, and Figure 5.10 in PG solution. The $Me_2SO$ was added because of successful chondrocyte recovery by Muldrew et. al. (66) in sheep articular cartilage with a similar protocol. These three samples exhibited matrix disruption similar to Figures 5.4, 5.5, 5.6, and 5.7 indicating extra water in the perilacunar area. These results were similar for all solutions used. Cells in Figures 5.8 and 5.9 were very similar in appearance exhibiting severe cellular retraction from the sidewalls with minimal contact remaining. There was also the presence of only a small amount of intracellular ice due to the extensive contraction of the chondrocytes. The majority of cells in PG solution had similar appearances to those in Figure 5.8 and 5.9 but a portion of them had the appearance seen in Figure 5.10. These cells showed total dissociation from lacunar sidewalls with the cells compacted into small balls likely due to severe dehydration. No intracellular ice was seen in these cells.

Slow freezing from -5°C to -30°C at 1°C/min would result in increased ice formation leading to increased electrolyte concentration and increased efflux of water from the cells. As water exited the cells, they shrunk and pulled away from the sidewalls of the lacuna. Extremes in electrolyte concentrations would result in extremes of shrinkage and continued retraction from the sidewalls. As the shrinkage continued there was complete dissociation of the cell from the lacuna as seen with samples in PG solution (Figure 5.10).

This freezing protocol correlated with poor chondrocyte recovery as assessed by vital dye assays in previous work (publication pending). This poor result could be due to shrinkage past the minimal cell volume compatible with continued cell survival (24,25,46). Other explanations may be that retraction of the cell from the sidewall may damage the cellular membrane making it more susceptible to intracellular ice nucleation from extracellular ice than it would normally be (15,16,17). Once again this intracellular ice would be lethal to the cell. The excessive increase in electrolyte concentration (13), the rapid efflux of water from the cell in response to increased electrolyte concentration (25,26), excessive dehydration (10), or altered electrical currents (16) may be lethal to the cell.

This series of SEM reinforced and documented some theories in cryobiology. The morphology of fresh chondrocytes in cartilagenous matrix and the subsequent change in cellular appearance and matrix consistency with the addition of "penetrating" cryoprotectants of 1,2-propanediol and $Me_2SO$ was demonstrated. 1,2-propanediol use was based on previous work in our lab showing PG to have superior chondrocyte recovery when compared to $Me_2SO$ on thin slices of human AC using a similar freezing protocol (11). Our past experience using $Me_2SO$ on 10mm diameter human osteochondral dowels was unsuccessful in cryopreserving chondrocytes *in situ* using the protocol established by McGann and Muldrew (19,27) even though they achieved moderate success with approximately 60% chondrocyte recovery in ovine AC.

The cells did not respond to the penetration cryoprotectant as expected with a return to original cell volume. Although the cell characteristics in this study were generally the same with PG and $Me_2SO$ (Figures 5.9 and 5.10), there was increased cellular shrinkage when PG was used. This could be due to either less water removed from the cell with $Me_2SO$ or possibly some permeation of $Me_2SO$ into the cells with subsequent partial equilibration. Work by Muldrew et. al. on $Me_2SO$ penetration in AC in pigs showed 30 min of exposure should allow penetration of a minimum of 2mm in depth. No differences in cellular morphology was noted in any of the samples immersed in cryoprotectant solution at any distance from the surface of the cartilage. It is possible that the cellular shrinkage noted was due to inability of the cryoprotectant to penetrate the cartilagenous matrix and its resultant effect as a nonpenetrating cryoprotectant with subsequent osmotic effects. Another possibility may be that even with diffusion of the cryoprotectant through the matrix, the cells may not respond as they do in isolation. Most work done on chondrocytes has been performed on isolated cells after dissolution of the matrix as by Schachar and McGann (35). The chemical and physical dissolution of the matrix may alter the cellular membrane permeability characteristics. This could explain the differences seen in isolated chondrocytes versus the results of chondrocytes *in situ.*

The SEM of samples after cooling to -5°C and extracellular ice nucleation demonstrated the effect of extracellular ice with its subsequent increase in electrolyte concentration and water efflux from the cells. The SEM consistently showed cellular shrinkage and perilacunar water consistent with this theory. Also noted was the consistently smaller sized chondrocytes in PG compared with those in PBS after the same procedures were performed. Intracellular ice formation as a lethal mechanism in chondrocyte freezing was also reinforce by the similar appearance of cells after cooling only to -5°C when compared to those frozen directly into LN, the negative control. The appearance of intracellular ice was consistent with chondrocyte death during this protocol.

The samples cooled to -30°C were also readily explicable by cryobiological principles. Once again the electrolyte theory with its accompanying solution effect was reaffirmed. The increased ice formation caused the electrolyte concentration to increase with a corresponding increase in the water efflux followed by cellular shrinkage. At the -30°C temperature level the cell shrinkage may have been too severe for chondrocyte recovery.

These results have revealed that more work needs to be done in the area of cryoprotection solutions and their penetration into intact cartilage. There has been reaffirmation of common cryobiological theories regarding the solution effects as well as increased evidence of lethal damage due to intracellular ice formation. It is clear that cryopreserving intact human AC is a difficult process and it is likely that information gathered from its study will be applicable to other human tissues.

## 5.5 ACKNOWLEDGMENTS

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## 5.7 FIGURES

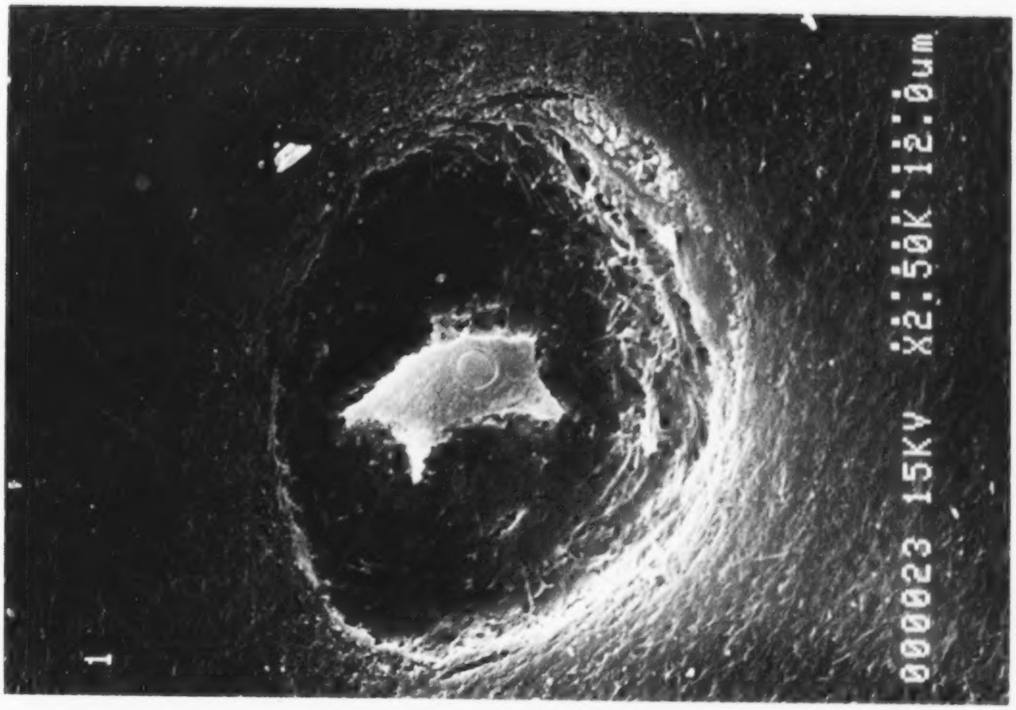


Figure 5.1 Scanning electron micrograph of fresh control in PBS solution. Note homogenous matrix and confluent cell structure completely filling the lacunae without breaks in sidewall continuity or intracellular disruption.

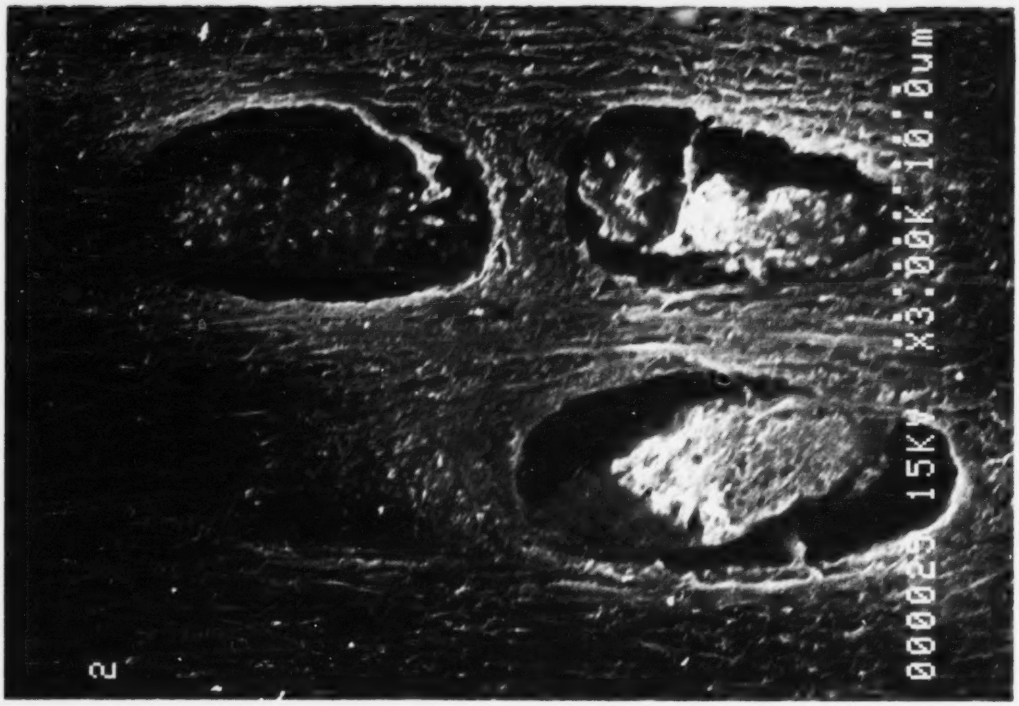


Figure 5.2 Scanning electron micrograph of fresh control in 1,2-propanediol solution. Note generally homogenous matrix with small amount of perilacunar water collection. The cells are intact without intracellular disruption but have circumferential retraction from the lacunar sidewalls.

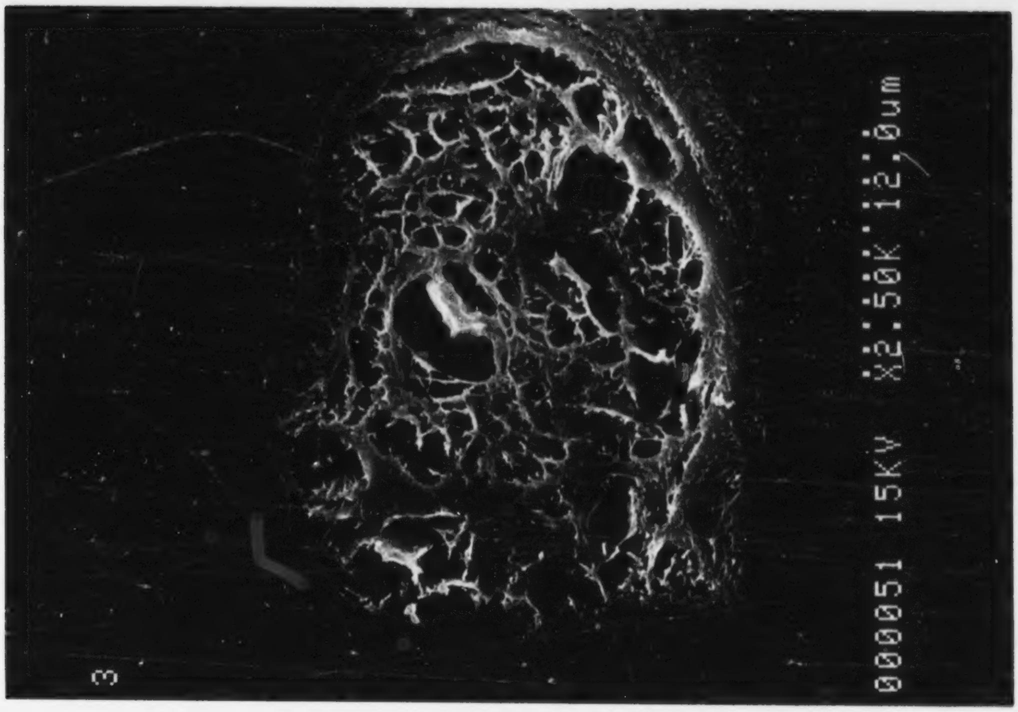


Figure 5.3 Negative control of sample in PBS plunged directly into LN from room temperature. Note the homogenous matrix appearance and the cell completely fills the lacunae with no evidence of cellular shrinkage. There is extensive intracellular disruption with multiple holes present indicating extensive intracellular ice formation.

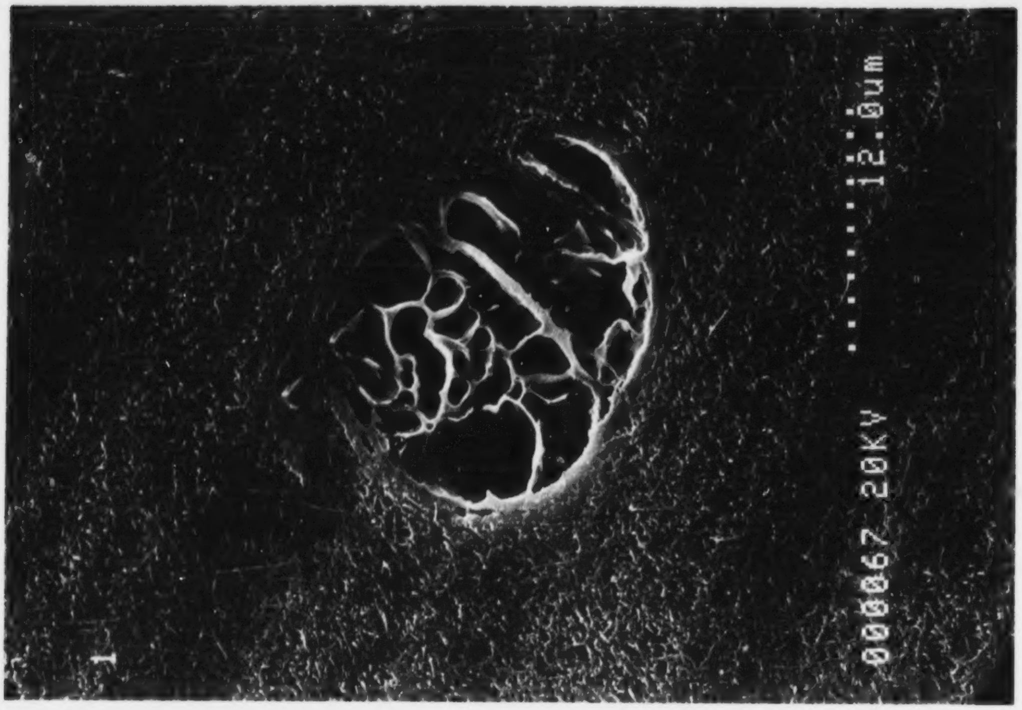


Figure 5.4 Sample in PBS cooled down to -5°C and then plunged into LN. Matrix exhibits little perilacunar ice and cell has not shrunk to any significant extent. There are extensive intracellular holes indicating extensive intracellular ice formation.

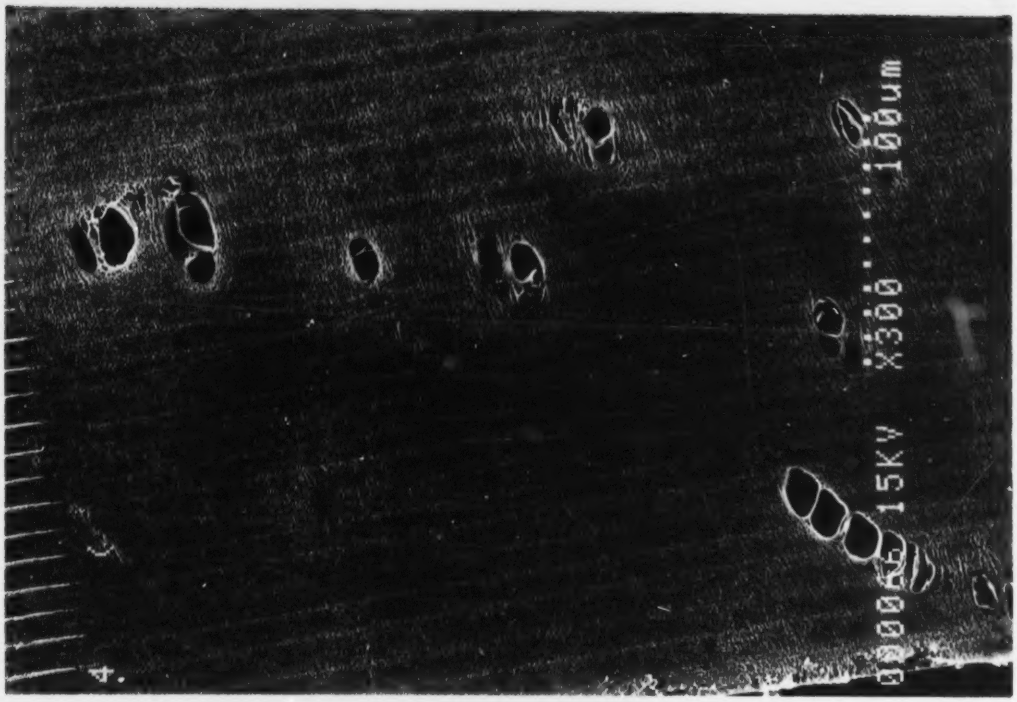

00005 15KV X300 100um
4

Figure 5.5 Sample as in Figure 4 with lower magnification. Note the small amount of perilacunar collections of water visible with homogenous appearance of the remaining matrix.

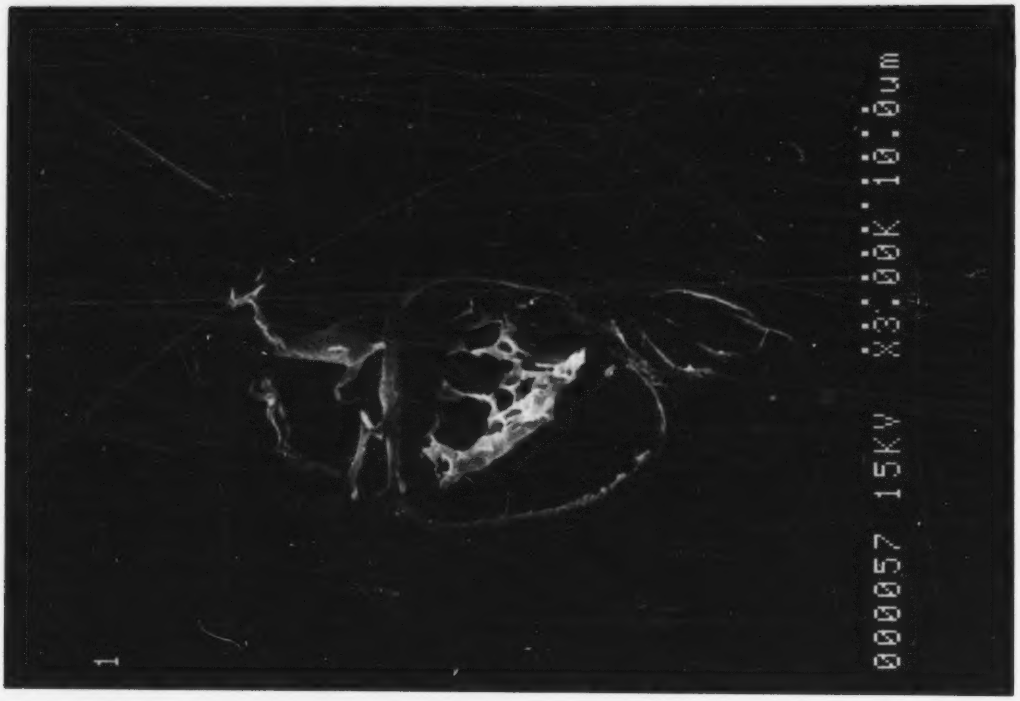
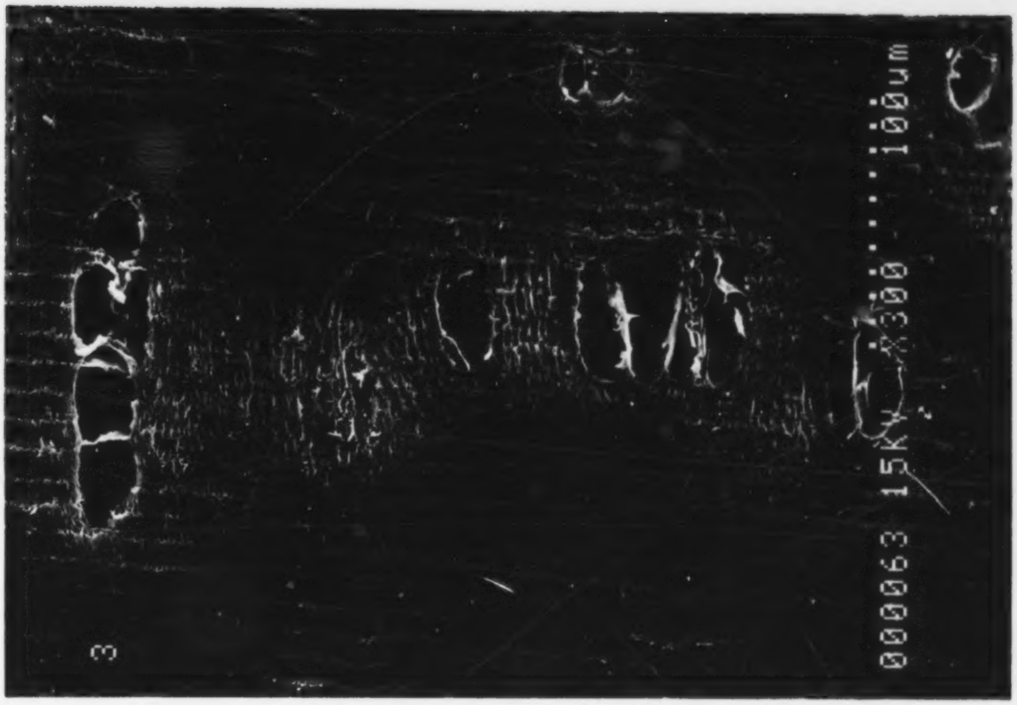

000057 15KV X3.00K 10.0um
1

Figure 5.6 Sample in 1,2-propanediol cooled down to -5°C and then plunged into LN. The cell is retracted from the sidewalls to a moderate extent and there are multiple holes indicating intracellular ice formation.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3

Figure 5.7 Sample as in Figure 6 with lower magnification. Note the increase collections of water in the matrix most concentrated in the perilacunar and adjacent areas.

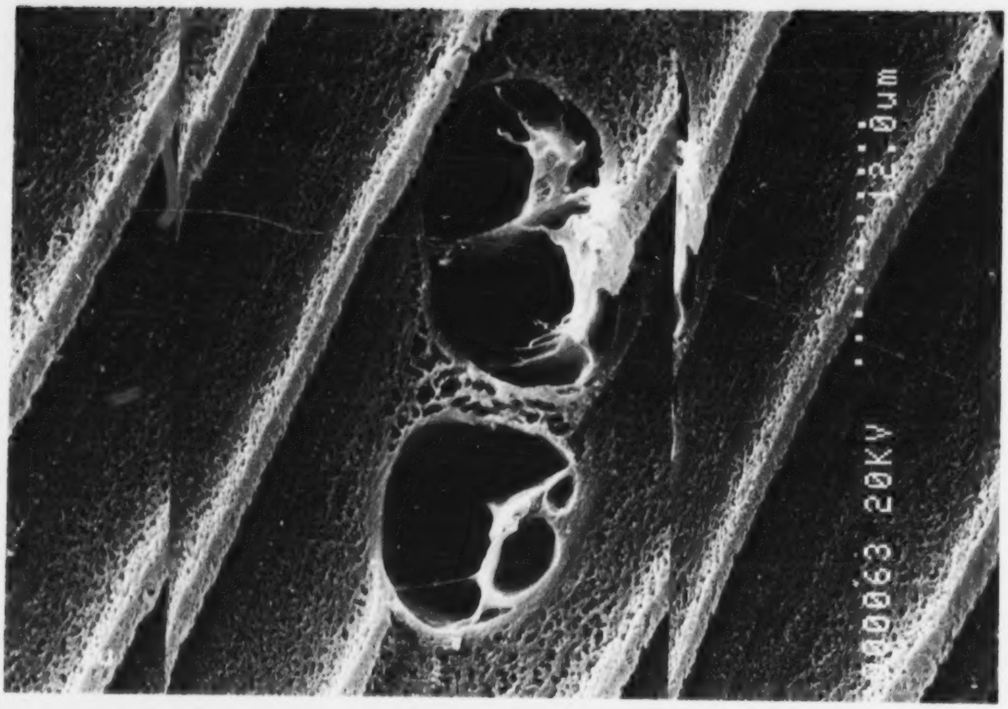

Figure 5.8 Sample in PBS slowly cooled to -30°C and then plunged into LN. The matrix again exhibits increased perilacunar water collections and a grainy appearance. The cells are markedly contracted with only a few strands of cytoplasm clinging to the sidewalls. There are some intracellular pockets formed indicating some intracellular ice formation.

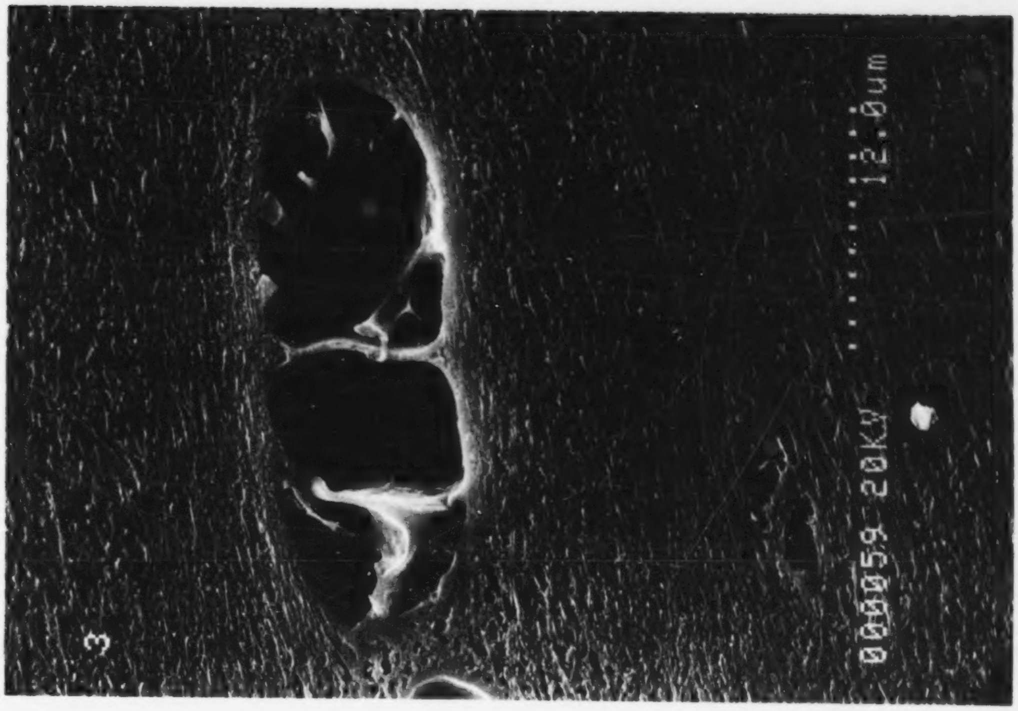

Figure 5.9 Sample in $Me_2SO$ cooled to -30°C and plunged into LN. Similar appearance to Figure 5.8 with grainy appearance to the matrix throughout. The cells are markedly contracted with few sidewall connections. Minimal intracellular ice is noted.

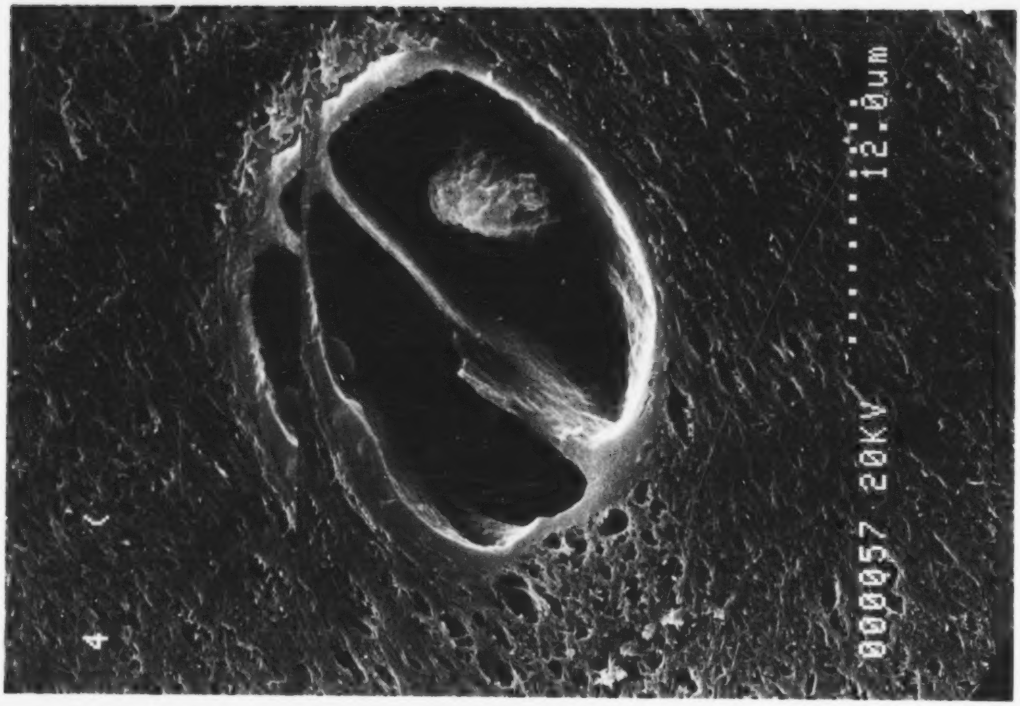

Figure 5.10 Sample in 1,2-propanediol cooled to -30°C and plunged into LN. Similar matrix appearance to Figures 5.8 and 5.9 but with a coarser grainy appearance. The cells is extremely contracted with no sidewall attachments. No intracellular ice formation is noted in the cell.

# CHAPTER 6

## 6.1 CONCLUSION

Freezing protocols introduce many differing cellular environments including altered electrolyte concentrations, cryoprotectant and ice exposure, significant solute and water fluxes and changes in cell membrane configuration among other less well defined conditions. Understanding of all conditions experienced by the cells would allow modification of existing protocols to compensate for adverse conditions.

Much work has been done using different cryoprotectants but the majority has been with $Me_2SO$ and glycerol. 1,2-propanediol has shown superior effect when used in direct comparison with these cryoprotectants under certain conditions in intact human articular cartilage. These results suggest alternatives to commonly used cryoprotectants may be beneficial during freezing procedures and that the optimal cryoprotectant for intact human AC requires definitive documentation.

Isolated chondrocytes have been successfully cryopreserved but the interaction between the cells, cartilage matrix, and cryoprotectant agent alters the response to freezing in intact AC. Events such as extracellular ice nucleation and prolonged exposure to ice induce lethal events to chondrocytes *in situ* which do not occur to cells in isolation. The changes to chondrocytes that occur with these events require identification to prevent lethal injury from occurring. The ability to proceed past extracellular ice nucleation with minimal cell damage may allow sufficient cellular dehydration to permit rapid freezing in LN without subsequent intracellular ice formation.

Scanning electron microscopy in combination with freeze substitution is useful to document *in situ* cellular morphology. Baseline information has been gathered describing cells and cartilagenous matrix under control and various freezing conditions. Initial information described water movement during freezing, including cellular shrinkage in response to freezing and increased electrolyte concentrations, that concurs with accepted cryobiologic explanations of events that occur with freezing. The distribution of water in the cell and matrix is important information that may provide insight to the actions of cryoprotectants and the conditions that are deleterious to cells.

Identification of cryoprotectant interaction with the chondrocytes and the cartilagenous matrix is a vital component to successful cryopreservation. Unexpectantly cellular shrinkage was noted with addition of cryoprotectant introducing the possibility of altered cryoprotectant interaction within the cartilagenous matrix. Further study of the interaction of the cryoprotectant within the cartilagenous matrix is important with focus on penetration ability and permeation kinetics with respect to *in situ* chondrocytes. Studies comparing varying lengths of exposure to cryoprotectants will provide information regarding penetration properties of each cryoprotectant into the matrix. Other alternatives such as different penetrating and nonpenetrating cryoprotectants may require investigation and can be used as comparison tools to help clarify the kinetics of the penetrating cryoprotectants.

Other important elements for successful cryopreservation include documentation of points of chondrocyte injury and the mechanism of this injury.

Events such as with extracellular ice nucleation and prolonged exposure to ice cause chondrocyte death in intact cartilage. What lethal event occurs during these two steps? What can be done to protect against these events? To answer these questions, changes that occur to chondrocytes *in situ* must be documented. Studies utilizing SEM and TEM may be useful to characterize morphologic changes that occur to the cells during these injurious times. Identification of the specific cause of damage, whether due to membrane alterations, intracellular ice, extracellular ice, or other mechanisms yet not identified would allow measures to avoid these circumstances.

Initial studies using animal models are important to develop ideas and test theories. This research demonstrated that prior to clinical application of proposed protocols to humans, it is essential to definitively reproduce results on human experimental models. Although gross appearances may be similar it is likely that there are more specific species dependent factors that come into play. These factors must be ruled out prior to clinical human use.

Events associated with significant cell damage require identification and subsequent clarification of mechanisms of injury. Comparison studies can help identify points of lethal injury such as that which occurs with extracellular ice nucleation and prolonged exposure to ice. Further descriptive studies documenting cell morphology under these circumstances may help delineate the mechanism of injury and aid in the development of protocols to avoid these situations. The majority of work in cryopreservation of AC has been empirical. The work performed here has provided a basis to expand research on intact human AC on a scientific level which may lead to consistent successful cryopreservation of intact human articular cartilage.